

The background of the cover is a composite of two microscopic images of lactic acid bacteria. The top half features a dark green background with numerous rod-shaped bacteria, some of which are in focus and show internal structures. The bottom half features a lighter, yellowish-green background with a dense cluster of similar rod-shaped bacteria, also showing some internal detail.

# Biotechnology of Lactic Acid Bacteria

**NOVEL APPLICATIONS**

**Fernanda Mozzi, Raúl R. Raya and Graciela M. Vignolo**

**EDITORS**



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# **Biotechnology of Lactic Acid Bacteria**

## *Novel Applications*

# Biotechnology of Lactic Acid Bacteria *Novel Applications*

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Editors

**Fernanda Mozzi**

*Centro de Referencia para Lactobacilos (CERELA)-CONICET  
San Miguel de Tucumán, Argentina*

**Raúl R. Raya**

*Centro de Referencia para Lactobacilos (CERELA)-CONICET  
San Miguel de Tucumán, Argentina*

**Graciela M. Vignolo**

*Centro de Referencia para Lactobacilos (CERELA)-CONICET  
San Miguel de Tucumán, Argentina*



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# Contributors

## Editors

**Fernanda Mozzi.** Centro de Referencia para Lactobacilos (CERELA)-CONICET. Chacabuco 145, T4000ILC San Miguel de Tucumán, Argentina. fmozzi@cerela.org.ar

**Raúl R. Raya.** Centro de Referencia para Lactobacilos (CERELA)-CONICET. Chacabuco 145, T4000ILC San Miguel de Tucumán, Argentina. rraya@cerela.org.ar

**Graciela M. Vignolo.** Centro de Referencia para Lactobacilos (CERELA)-CONICET. Chacabuco 145, T4000ILC San Miguel de Tucumán, Argentina. vignolo@cerela.org.ar

## Contributors

**Analía G. Abraham.** Centro de Investigación y Desarrollo en Criotecnología de Alimentos (CIDCA)-CONICET. Facultad de Ciencias Exactas (UNLP) 47 y 116, 1900 La Plata, Buenos Aires, Argentina. aga@biol.unlp.edu.ar

**Laura Aguirre.** Centro de Referencia para Lactobacilos (CERELA)-CONICET. Chacabuco 145, T4000ILC San Miguel de Tucumán, Argentina. Cátedra de Bioquímica, Facultad de Medicina, Universidad Nacional de Tucumán, Tucumán, Argentina. laguirre@cerela.org.ar

**Tamara Aleksandrak-Piekarczyk.** Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Pawlinskiego 5a, 02-106 Warsaw, Poland. tamara@ibb.waw.pl

**Gladis Susana Alvarez.** Centro de Referencia para Lactobacilos. (CERELA)-CONICET. Chacabuco 145, T4000ILC San Miguel de Tucumán, Argentina. Facultad de Bioquímica, Química y Farmacia. Universidad Nacional de Tucumán. Ayacucho 491, T4000ILC San Miguel de Tucumán, Argentina. salvarez@cerela.org.ar

**Pablo Álvarez-Martín.** Departamento de Microbiología y Bioquímica, Instituto de Productos Lácteos de Asturias (CSIC), Carretera de Infesto s/n, 33300-Villaviciosa, Asturias, Spain. pablo-alvarez@ipla.csic.es

**M. Andrea Azcarate-Peril\*.** Department of Food, Bioprocessing, and Nutrition Sciences, and Southeast Dairy Foods Research Center, North Carolina State University, Raleigh, North Carolina. Present address: Department of Cell and Molecular Physiology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina at Chapel Hill, Chapel Hill, NC 27599-7545. azcarate@med.unc.edu

**Vasco Azevedo\*.** Laboratório de Genética Celular e Molecular, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais (ICB/UFMG), Belo Horizonte-MG, Brasil. vasco@icb.ufmg.br

**Jacek Bardowski.** Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Pawinskiego 5a, 02-106 Warsaw, Poland. jacek@ibb.waw.pl

**Luis G. Bermúdez-Humarán.** Unité d'Écologie et Physiologie du Système Digestif (UEPSD), INRA Centre de Recherche de Jouy, Domaine de Vilvert 78352, Jouy-en-Josas, cedex France. luis.bermudez@jouy.inra.fr

**Ana Binetti.** Instituto de Lactología Industrial (INLAIN), Universidad Nacional del Litoral-CONICET, 3000 Santa Fe, Argentina. anabinetti@fiq.unl.edu.ar

**Nicholas Camu.** Research Group of Industrial Microbiology and Food Biotechnology (IMDO), Department of Applied Biological Sciences and Engineering, Vrije Universiteit Brussel, Pleinlaan 2, B-1050 Brussels, Belgium. nicholas.camu@vub.ac.be

**Domenico Carminati.** Agricultura Research Council. Research Centre for Forage and Dairy Productions (CRA-FLC), Research Unit "Dairy Productions", Via Lombardo 11, 26900 Lodi, Italy. dcarminati@ilclodi.it

**Marie-Christine Champomier-Vergès.** INRA Centre de Recherche de Jouy (UR309) Unité FLEC, Domaine de Vilvert 78350, Jouy-en-Josas, cedex France. marie-christine.champomier-verges@jouy.inra.fr

**Gyu-Sung Cho.** Max Rubner-Institute, Federal Research Institute for Nutrition and Food, Department of Safety and Quality of Fruits and Vegetables, Haid-und-Neu-Strasse 9, 76131 Karlsruhe, Germany. guy.cho@mri.bund.de

**Pier S. Cocconcelli.** Istituto di Microbiologia, Università Cattolica del Sacro Cuore, via Emilia Parmense 84, 29100 Piacenza, Italy. pier.cocconcelli@unicatt.it

**Barry Collins.** Department of Microbiology, University College Cork, Cork, Ireland. bac@student.ucc.ie

**Paul D. Cotter\*.** Moorepark Food Research Centre; Alimentary Pharmabiotic Centre, Teagasc, Moorepark, Fermoy, Cork, Ireland. p.cotter@ucc.ie

**Graciela L. De Antoni\*.** Centro de Investigación y Desarrollo en Criotecnología de Alimentos (CIDCA)-CONICET. Facultad de Ciencias Exactas (UNLP) 47 y 116, 1900 La Plata, Buenos Aires, Argentina. gdeantoni@biol.unlp.edu.ar

**Alejandra de Moreno de LeBlanc.** Centro de Referencia para Lactobacilos (CERELA)-CONICET. Chacabuco 145, T4000ILC San Miguel de Tucumán, Argentina. demoreno@cerela.org.ar

**Luc De Vuyst\*.** Research Group of Industrial Microbiology and Food Biotechnology (IMDO), Faculty of Sciences and Bio-Engineering Sciences, Vrije Universiteit Brussel, Pleinlaan 2, B-1050 Brussels, Belgium. ldvuyst@vub.ac.be

**Cecilia Dogi.** Centro de Referencia para Lactobacilos (CERELA)-CONICET. Chacabuco 145, T4000ILC San Miguel de Tucumán, Argentina. cdogi@cerela.org.ar

**Silvina Fadda\*.** Centro de Referencia para Lactobacillos (CERELA)-CONICET. Chacabuco 145, T4000ILC San Miguel de Tucumán, Argentina. sfadda@cerela.org.ar

**María Fernández.** Departamento de Tecnología y Biotecnología, Instituto de Productos Lácteos de Asturias (CSIC), Carretera de Infesto s/n, 33300-Villaviciosa, Asturias, Spain. mfernandez@ipla.csic.es

**Pasquale Ferranti.** Dipartimento di Scienza degli Alimenti, University of Naples Federico II, Parco Gussone, Portici I- 80055, Italy. ferranti@unina.it

**Graciela Font de Valdez\*.** Centro de Referencia para Lactobacilos (CERELA)-CONICET. Chacabuco 145, T4000ILC San Miguel de Tucumán, Argentina. Cátedra de Microbiología Superior, Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán. Ayacucho 491, T4000ILC San Miguel de Tucumán, Argentina. gfont@cerela.org.ar

**Cecilia Fontana.** Centro Ricerche Biotecnologiche, Università Cattolica del Sacro Cuore, via Milano 24, 26100 Cremona, Italy. cecilia.fontana@unicatt.it

**Charles M.A.P. Franz\*.** Max Rubner-Institute, Federal Research Institute for Nutrition and Food, Department of Safety and Quality of Fruits and Vegetables, Haid-und-Neu-Strasse 9, 76131 Karlsruhe, Germany. Charles.Franz@mri.bund.de

**Antonio Gálvez.** Departamento de Ciencias de la Salud. Área de Microbiología. Facultad de Ciencias Experimentales. Universidad de Jaén. 23071-Jaén, Spain. agalvezp@yahoo.es

**Pilar García.** Instituto de Productos Lácteos de Asturias (CSIC), Carretera de Infesto s/n, 33300-Villaviciosa, Asturias, Spain.pgarcia@ipla.csic.es

**Marisa S. Garro.** Centro de Referencia para Lactobacilos (CERELA)-CONICET. T4000ILC San Miguel de Tucumán, Argentina. mgarro@cerela.org.ar

**Graciela L. Garrote.** Centro de Investigación y Desarrollo en Criotecología de Alimentos (CIDCA)-CONICET. Facultad de Ciencias Exactas (UNLP) 47 y 116, 1900 La Plata, Buenos Aires, Argentina. ggarrote@biol.unlp.edu.ar

**Carla L. Gerez.** Centro de Referencia para Lactobacilos (CERELA)-CONICET. Chacabuco 145, T4000ILC San Miguel de Tucumán, Argentina. clgerez@cerela.org.ar

**Giorgio Giraffa.** Agricoltura Research Council. Research Centre for Forage and Dairy Productions (CRA-FLC), Research Unit “Dairy Productions”, Via Lombardo 11, 26900 Lodi, Italy. ggiraffa@ilclodi.it

**Elvira María Hebert\*.** Centro de Referencia para Lactobacilos (CERELA)-CONICET. Chacabuco 145, T4000ILC San Miguel de Tucumán, Argentina. ehebert@cerela.org.ar

**Colin Hill\*.** Department of Microbiology, University College Cork, Cork, Ireland. Alimentary Pharmabiotic Centre, University College Cork, Ireland. c.hill@ucc.ie

**Wilhelm H. Holzapfel.** Handong Global University, School of Life and Food Sciences, Pohang, Gyeongbuk, 791-708, Korea. wilhelm@handong.edu

**María Silvina Juárez Tomás.** Centro de Referencia para Lactobacilos. (CERELA)-CONICET. Chacabuco 145. T4000ILC. San Miguel de Tucumán, Argentina. msjuarez@cerela.org.ar

**Todd R. Klaenhammer.** Department of Food, Bioprocessing, and Nutrition Sciences, and Southeast Dairy Foods Research Center, North Carolina State University, Raleigh, North Carolina. trk@unity.ncsu.edu

**Magdalena Kowalczyk.** Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Pawinskiego 5a, 02-106 Warsaw, Poland. mk@ibb.waw.pl

**Philippe Langella.** Unité d'Écologie et Physiologie du Système Digestif (UEPSD), INRA Centre de Recherche de Jouy, Domaine de Vilvert 78352, Jouy-en-Josas, cedex France. philippe.langella@jouy.inra.fr



**Jean Guy LeBlanc.** Centro de Referencia para Lactobacilos (CERELA)-CONICET. Chacabuco 145, T4000ILC San Miguel de Tucumán, Argentina. leblanc@cerela.org.ar

**Timothy Lefeber.** Research Group of Industrial Microbiology and Food Biotechnology (IMDO), Department of Applied Biological Sciences and Engineering, Vrije Universiteit Brussel, Pleinlaan 2, B-1050 Brussels, Belgium. timothy.lefeber@vub.ac.be

**Graciela Lorca.** Department of Microbiology and Cell Science. University of Florida. Gainesville, FL32608, USA. glorca@ufl.edu

**Carolina Maldonado Galdeano.** Centro de Referencia para Lactobacilos (CERELA)-CONICET. Chacabuco 145, T4000ILC San Miguel de Tucumán, Argentina. Cátedra de Inmunología. Instituto de Microbiología. Facultad de Bioquímica, Química y Farmacia. Universidad Nacional de Tucumán. Ayacucho 491, T4000ILC, San Miguel de Tucumán, Argentina. cmaldo@cerela.org.ar

**José Marazza.** Centro de Referencia para Lactobacilos (CERELA)-CONICET. Chacabuco 145, T4000ILC San Miguel de Tucumán, Argentina. jmarazza@cerela.org.ar

**Baltasar Mayo\*.** Departamento de Microbiología y Bioquímica, Instituto de Productos Lácteos de Asturias (CSIC), Carretera de Infesto s/n, 33300-Villaviciosa, Asturias, Spain. baltasar.mayo@ipla.csic.es

**Marcela Medina.** Centro de Referencia para Lactobacilos (CERELA)-CONICET. Chacabuco 145, T4000ILC San Miguel de Tucumán, Argentina. Facultad de Bioquímica, Química y Farmacia. Universidad Nacional de Tucumán. Ayacucho 491, T4000ILC San Miguel de Tucumán, Argentina. mmedina@cerela.org.ar

**Anderson Miyoshi\*.** Laboratório de Genética Celular e Molecular, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais (ICB/UFGM), Belo Horizonte-MG, Brasil. miyoshi@icb.ufmg.br

**Verónica Molina.** Centro de Referencia para Lactobacilos (CERELA)-CONICET. Chacabuco 145, T4000ILC San Miguel de Tucumán, Argentina. vmolina@cerela.org.ar

**Fernanda Mozzi\*.** Centro de Referencia para Lactobacilos (CERELA)-CONICET. Chacabuco 145, T4000ILC San Miguel de Tucumán, Argentina. fmozzi@cerela.org.ar

**María E. Fátima Nader-Macías\*.** Centro de Referencia para Lactobacilos (CERELA)-CONICET. Chacabuco 145. T4000ILC. San Miguel de Tucumán, Argentina. fnader@cerela.org.ar

**Anphong Nguyen.** Division of Biological Sciences, University of California at San Diego, La Jolla, CA92093, USA. aln012@ucsd.edu

**Zoe Papalexandratou.** Research Group of Industrial Microbiology and Food Biotechnology (IMDO), Department of Applied Biological Sciences and Engineering, Vrije Universiteit Brussel, Pleinlaan 2, B-1050 Brussels, Belgium. zoe.papalexandratou@vub.ac.be

**Gabriela Perdigon\*.** Centro de Referencia para Lactobacilos (CERELA)-CONICET. Chacabuco 145, T4000ILC San Miguel de Tucumán, Argentina. Cátedra de Inmunología. Instituto de Microbiología. Facultad de Bioquímica, Química y Farmacia. Universidad Nacional de Tucumán. Ayacucho 491, T4000ILC San Miguel de Tucumán, Argentina. perdigon@cerela.org.ar

**Andrea Quiberoni.** Instituto de Lactología Industrial (INLAIN), Universidad Nacional del Litoral-CONICET, 3000 Santa Fe, Argentina. [aquibe@fiq.unl.edu.ar](mailto:aquibe@fiq.unl.edu.ar)

**Raúl R. Raya.** Centro de Referencia para Lactobacilos (CERELA)-CONICET. Chacabuco 145, T4000ILC San Miguel de Tucumán, Argentina. [rraya@cerela.org.ar](mailto:rraya@cerela.org.ar)

**Lakshmi Reddy.** Division of Biological Sciences, University of California at San Diego, La Jolla, CA92093, USA. [breddy@glendale.edu](mailto:breddy@glendale.edu)

**Jorge Reinheimer\*.** Instituto de Lactología Industrial (INLAIN), Universidad Nacional del Litoral-CONICET, 3000 Santa Fe, Argentina. [jorreinh@fiq.unl.edu.ar](mailto:jorreinh@fiq.unl.edu.ar)

**Pierre Renault\*.** Génétique Microbienne, INRA Centre de Recherche de Jouy, Domaine de Vilvert 78352, Jouy-en-Josas, cedex France. [pierre.renault@jouy.inra.fr](mailto:pierre.renault@jouy.inra.fr)

**Tom Rimaux.** Research Group of Industrial Microbiology and Food Biotechnology (IMDO), Faculty of Sciences and Bio-Engineering Sciences, Vrije Universiteit Brussel, Pleinlaan 2, B-1050 Brussels, Belgium. [tom.rimaux@vub.ac.be](mailto:tom.rimaux@vub.ac.be)

**Ana Rodríguez González\*.** Departamento de Tecnología y Biotecnología de Productos lácteos. Instituto de Productos Lácteos de Asturias (CSIC), Carretera de Infesto s/n, 33300-Villaviciosa, Asturias, Spain. [anarguez@ipla.csic.es](mailto:anarguez@ipla.csic.es)

**Graciela Rollán.** Centro de Referencia para Lactobacilos (CERELA)-CONICET. Chacabuco 145, T4000ILC San Miguel de Tucumán, Argentina. [rollan@cerela.org.ar](mailto:rollan@cerela.org.ar)

**R. Paul Ross.** Alimentary Pharmabiotic Centre, University College Cork, Ireland. Dairy Products Research Centre, Teagasc, Moorepark, Fermoy, Co. Cork, Ireland. [paul.ross@teagasc.ie](mailto:paul.ross@teagasc.ie)

**Lucila Saavedra.** Centro de Referencia para Lactobacilos (CERELA)-CONICET. Chacabuco 145, T4000ILC San Miguel de Tucumán, Argentina. [lucila@cerela.org.ar](mailto:lucila@cerela.org.ar)

**Milton H. Saier, Jr.\*.** Division of Biological Sciences, University of California at San Diego, La Jolla, CA92093, USA. [msaier@ucsd.edu](mailto:msaier@ucsd.edu)

**Marcela Santiago Pacheco de Azevedo.** Laboratório de Genética Celular e Molecular, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais (ICB/UFMG), Belo Horizonte-MG, Brasil. [marcelinhapsa@yahoo.com.br](mailto:marcelinhapsa@yahoo.com.br)

**Graciela Savoy de Giori\*.** Centro de Referencia para Lactobacilos (CERELA)-CONICET. Chacabuco 145, T4000ILC San Miguel de Tucumán, Argentina. Cátedra de Microbiología Superior, Universidad Nacional de Tucumán. Ayacucho 491, T4000ILC San Miguel de Tucumán, Argentina. [gsavoy@cerela.org.ar](mailto:gsavoy@cerela.org.ar)

**Fernando Sesma\*.** Centro de Referencia para Lactobacilos (CERELA)-CONICET. Chacabuco 145, T4000ILC San Miguel de Tucumán, Argentina. [fesma@cerela.org.ar](mailto:fesma@cerela.org.ar)

**Clara Silva de Ruiz.** Facultad de Bioquímica, Química y Farmacia. Universidad Nacional de Tucumán. Ayacucho 491, T4000ILC San Miguel de Tucumán, Argentina. [csilva@fbqf.unt.edu.ar](mailto:csilva@fbqf.unt.edu.ar)

**Viviana Suárez.** Instituto de Lactología Industrial (INLAIN), Universidad Nacional del Litoral-CONICET, 3000 Santa Fe, Argentina. [vivisuar@fiq.unl.edu.ar](mailto:vivisuar@fiq.unl.edu.ar)

**Eric I. Sun.** Division of Biological Sciences, University of California at San Diego, La Jolla, CA92093, USA. [eisun@ucsd.edu](mailto:eisun@ucsd.edu)

**María Pía Taranto.** Centro de Referencia para Lactobacilos (CERELA)-CONICET. Chacabuco 145, T4000ILC San Miguel de Tucumán, Argentina. ptaranto@cerela.org.ar

**María Inés Torino.** Centro de Referencia para Lactobacilos (CERELA)-CONICET. Chacabuco 145, T4000ILC San Miguel de Tucumán, Tucumán, Argentina. mitorino@cerela.org.ar

**John Tseng.** Division of Biological Sciences, University of California at San Diego, La Jolla, CA92093, USA.

**Graciela M. Vignolo\*.** Centro de Referencia para Lactobacilos (CERELA)-CONICET. Chacabuco 145, T4000ILC Tucumán, Argentina. vignolo@cerela.org.ar

**Gino Vrancken.** Research Group of Industrial Microbiology and Food Biotechnology (IMDO), Faculty of Sciences and Bio-Engineering Sciences, Vrije Universiteit Brussel, Pleinlaan 2, B-1050 Brussels, Belgium. gino.vrancken@vub.ac.be

**Ming-Ren Yen.** Division of Biological Sciences, University of California at San Diego, La Jolla, CA92093, USA. mryen@biomail.ucsd.edu

**Monique Zagorec.** INRA (UR309) Unité FLEC, INRA Centre de Recherche de Jouy, Domaine de Vilvert 78350, Jouy-en-Josas, cedex, France. monique.zagorec@jouy.inra.fr

**\*Corresponding author**

## Preface

Lactic acid bacteria (LAB) are food-grade microorganisms that play an essential role in the fermentation of animal and vegetable raw materials. Their ability to ferment carbohydrates and to a lesser extent to degrade the proteins and the fat present in those substrates leads to the synthesis of a broad range of compounds, such as organic acids, peptides, antimicrobial and aromatic compounds, and exopolysaccharides. Vitamins, low-calorie sugars, and bioactive peptides are also produced by LAB. These metabolites may contribute to the organoleptic, technological, and nutritional characteristics of fermented food products. Moreover, certain LAB strains, the so-called probiotics, display beneficial health properties and may be involved in the production of specific functional foods.

Ongoing research on LAB and their applications is at a very exciting stage worldwide. This book encompasses a wide range of topics in the area of LAB, including basic issues (metabolism, biodiversity, and transport systems), comprehensive information on new advanced approaches (genomics and proteomics), human-health LAB-related aspects, and LAB safety, as well as traditional and novel biotechnological applications. We feel this book may serve as an essential reference for established researchers and scientists, doctoral and postdoctoral students, university professors and instructors, and food technologists working on food microbiology, physiology, and biotechnology of LAB.



Fernanda Mozzi, Graciela M. Vignolo, and Raúl R. Raya

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# **Biotechnology of Lactic Acid Bacteria**

## *Novel Applications*

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Edited by Fernanda Mozzi, Raúl R. Raya and Graciela M. Vignolo

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# Chapter 1

## Updates in the Metabolism of Lactic Acid Bacteria

Baltasar Mayo, Tamara Aleksandrzyk-Piekarczyk, María Fernández, Magdalena Kowalczyk, Pablo Álvarez-Martín, and Jacek Bardowski

*Lactic acid bacteria (LAB) are fermentative bacteria naturally dwelling in or intentionally added to nutrient-rich environments where carbohydrates and proteins are usually abundant. The efficient use of nutrients and the concomitant production of lactic acid during growth endow LAB with remarkable selective advantages in the diverse ecological niches they inhabit. Besides lactic acid, LAB metabolism produces a variety of compounds, such as diacetyl, acetoin and 2-3-butanediol from the utilization of citrate, and a vast array of volatile compounds and bioactive peptides from the catabolism of amino acids. The enzymatic reactions of LAB metabolism further modify the organoleptic, rheological, and nutritive properties of the raw materials, giving rise to final fermented products. Last decade witnessed an impressive amount of data on several aspects of LAB physiology and genetics. The latest knowledge was gathered through sequencing and analysis of LAB genomes, and the subsequent use of post-genomic techniques, such as proteomics, comparative genome hybridization, transcriptomics, and metabolomics. Manipulation of the metabolic pathways of LAB to improve their efficiency in various industrial applications (as starters, adjunct cultures, and probiotics) was undertaken soon after the development of early engineering tools. The availability of complete genome sequences of different LAB species and strains has expanded our ability to further study LAB metabolism from a global perspective, strengthening a full exploitation of LAB's metabolic potential.*

### 1.1. Introduction

Lactic acid bacteria (LAB) encompass a heterogeneous group of microorganisms having as a common metabolic property the production of lactic acid as the majority end-product from the fermentation of carbohydrates (Carr et al. 2002). LAB are Gram (+), non-sporulating, catalase-negative, acid-tolerant, facultative anaerobic organisms. Except for a few species, LAB members are nonpathogenic organisms with a reputed Generally Recognized as Safe status. Typical LAB species belong to the genera *Lactobacillus*, *Lactococcus*, *Streptococcus*, *Pediococcus*, *Oenococcus*, *Enterococcus*, and *Leuconostoc*. Under a biochemical perspective LAB include both homofermenters, producing mainly lactic acid, and heterofermenters, which, apart from lactic acid, yield a large variety of fermentation products such as acetic acid, ethanol, carbon dioxide, and formic acid (Kleerebezem and Hugenholtz 2003). LAB are found in a large variety of nutrient-rich environments, including milk and dairy products, vegetable and plants, cereals, and meat and meat products. Many species are used for the manufacture and preservation of fermented feed and foods from raw agricultural materials in which they are either present as contaminants or deliberately added as starters in order to control the fermentations. Enzymatic activities of LAB contribute to the final organoleptic, rheological, and nutritional properties of fermented products (Leroy and de Vuyst 2004). LAB species are also commonly found

among the resident microbiota of the gastrointestinal tract and genitourinary tract of humans and animals (Eckburg et al. 2005; Marchesi and Shanahan 2007). In these environments LAB are considered essential components, playing a large variety of health-promoting functions, such as immunomodulation, intestinal integrity, and pathogen resistance (Vaughan et al. 2005). For such reasons strains of some species have traditionally been used as probiotics and added as functional bacteria in various food commodities (Ljungh and Wadström 2006). The commercial exploitation of LAB as starter and probiotic cultures is economically very significant. Consequently, research on their genetics, physiology, and applications has bloomed in the last 25 years (Wood and Warner 2003; Gasson and de Vos 2004).

This chapter addresses recent data on LAB metabolism related to their efficient utilization of niche- and product-related nutrients and the concomitant generation of desirable compounds for their industrial application. Modification of metabolic routes aimed to increase the efficacy of LAB in traditional fermentations, and their contribution to health and well-being will further be addressed. However, though important in dairy products, lipid metabolism, which has been only marginally studied in LAB (Collins et al. 2003; Hickey et al. 2006), will not be covered in this review. Additionally, the metabolism of nucleotides, obligatory metabolites for RNA and DNA synthesis and energy donors in many cellular processes, will not be discussed as well. In this last topic, readers are given an overview of the excellent recent work by Kilstrup et al. (2005).

## 1.2. Sugar Metabolism

Sugars are primary carbon and energy sources for LAB grown on substrates used for fermented food and feed production as well as in laboratory media. Many different transport systems involved in the uptake of carbohydrates, including PhosphoTransferase Systems (PTS), ATP-binding cassette (ABC), and Glycoside-Pentoside-Hexuronide transporters, exist in LAB (this book, Chapter 4). Polysaccharides before uptake have to be hydrolyzed; for example, starch is hydrolyzed either by  $\alpha$ -amylase into dextrins, which are subsequently hydrolyzed by an extracellular activity to maltose (Calderon Santoyo et al. 2003), or by debranching enzymes (Doman-Pytka et al. 2004). Monosaccharides entering the cell or liberated in the cytoplasm by hydrolysis of disaccharides enter glycolysis at the level of glucose-6P (G6P) or are processed by the Leloir pathway (Fig. 1.1D). In many strains of *Streptococcus thermophilus* only the glucose moiety of lactose is fermented, while the galactose moiety is excreted into the medium as a result of the weak transcription from *gal* promoters or mutations in the Leloir genes (Vaughan et al. 2001; de Vin et al. 2005; Fig. 1.1D). In *Lactococcus lactis* lactose transported by the PTS system is hydrolyzed and the galactose-6P moiety is transformed by the tagatose pathway, entering glycolysis at the level of triose phosphate. The initial metabolism of glucose, galactose, and lactose in *L. lactis* has been described by Coccagn-Bousquet et al. (2002) and Neves et al. (2005). Recently, it was postulated that in *L. lactis* IL1403 the metabolism of  $\beta$ -glucosides (cellobiose) and  $\beta$ -galactosides

**Figure 1.1.** Pathways of glucose metabolism. A. Homofermentative pathway; B. Mixed-acid metabolism; C. Heterofermentative pathway; D. Leloir pathway. Reactions are catalyzed by the following enzymes: 1, glucokinase (GLK); 2, glucose-phosphate isomerase (GPI); 3, phosphofructokinase (PFK); 4, fructose-bisphosphate aldolase (FBPA); 5, triose-phosphate isomerase (TPI); 6, glyceraldehyde-phosphate dehydrogenase (GAPDH); 7, phosphoglycerate kinase (PGK); 8, phosphoglycerate mutase (PMG); 9, enolase (ENO); 10, pyruvate kinase (PK); 11, lactate dehydrogenase (LDH); 12, pyruvate dehydrogenase (PDH); 13, pyruvate formate lyase (PFL); 14, acetaldehyde dehydrogenase (ACDH); 15, alcohol dehydrogenase (ADHE); 16, phosphotransacetylase (PTA); 17, acetate kinase (ACK); 18,  $\alpha$ -acetolactate synthase (ALS); 19,  $\alpha$ -acetolactate decarboxylase (ALD); 20, 2,3-butanediol dehydrogenase (BDH); 21, diacetyl reductase (DR); 22, glucose-6-P dehydrogenase (G6PDH); 23, 6-P-gluconate dehydrogenase (6PGDH); 24, ribulose-5-P-3-epimerase (RPPE); 25, D-xylulose-5P phosphoketolase (XPK); 26, galactokinase (GK); 27, galactose-1-P-uridylyltransferase (GPUULT); 28, UDP-galactose-1-epimerase (UDPE); 29, phosphoglucomutase (PGM).



(lactose) are interconnected (Aleksandrak-Piekarczyk et al. 2005; Kowalczyk et al. 2008) and, interestingly, lactose-P is hydrolyzed by BglS, a P- $\beta$ -glucosidase exhibiting P- $\beta$ -galactosidase activity (Aleksandrak-Piekarczyk et al. 2005).

Phosphorylated trehalose in *L. lactis* is split by the inorganic phosphate-dependent trehalose-6P phosphorylase (TrePP). TrePP catalyzes the conversion of trehalose-6P to  $\beta$ -glucose-1P ( $\beta$ -G1P) and G6P (Andersson et al. 2001).  $\beta$ -G1P is then reversibly converted into G6P by  $\beta$ -phosphoglucosyltransferase ( $\beta$ -PGM), which has been found to be essential in the catabolism of both trehalose and maltose (Levander et al. 2001; Andersson and R  dstr  m 2002a). This enzyme together with Pi-dependent maltose phosphorylase is involved in the degradation of maltose in *L. lactis* (Andersson and R  dstr  m 2002b). Hydrolysis of melibiose and raffinose by  $\alpha$ -galactosidase leads to the production of  $\alpha$ -galactose subunits, which are subsequently degraded by the enzymes of the tagatose-6P and Leloir pathways. Characterization and transcriptional analysis of the *aga-galKT* operon in *Lactococcus raffinolactis* has been presented by Boucher et al. (2003).

### 1.2.1. Homofermentation and Mixed-Acid Fermentation

Homofermentative LAB (*Lactococcus*, *Streptococcus*, *Pediococcus*, *Enterococcus*, and some species of *Lactobacillus*) ferment sugars by the Embden-Meyerhoff-Parnas (EMP) pathway to pyruvate, which is converted into lactic acid by lactate dehydrogenase (LDH; Fig. 1.1A). Two types of lactate isomers, L and D, can be produced by stereospecific NAD-dependent enzymes, L-LDH and D-LDH. Both enzymes have been found to be active in most lactobacilli, for example, in *Lactobacillus plantarum* (Ferain et al. 1996) and *Lactobacillus casei* (Viana et al. 2005a), whereas the single L-LDH in *Lactobacillus sakei* and *L. lactis* is complemented by a racemase or the protein product of the cryptic *ldhB* gene, respectively (Bongers et al. 2003; Gaspar et al. 2007).

Under certain conditions (carbon limitation, carbon excess of slowly metabolized sugars) the

homolactic metabolism can be shifted to a mixed-acid metabolism (Fig. 1.1B). This type of homofermentation is characterized by the production of formate, acetate, ethanol, and/or CO<sub>2</sub> in addition to lactate. There are several possible pathways for acetate production. Pyruvate can be metabolized anaerobically into acetate by pyruvate formate lyase (PFL), phosphotransacetylase (PTA), and acetate kinase (ACK) or aerobically by the pyruvate dehydrogenase complex, PTA, and ACK. In *Lact. plantarum*, the maximal acetate production is under aerobic conditions with glucose limitation. In this pathway acetate originates from lactate through the action of LDHs, pyruvate oxidase (POX), and ACK. Recently, the role of NAD-dependent LDHs (LdhD and LdhL), instead of the previously proposed NAD-independent LDHs (LoxD and LoxL), was confirmed during the stationary phase of aerobic growth in *Lact. plantarum* (Goffin et al. 2004). Two genes coding for PoxB and PoxF out of four *pox* genes in the genome are also involved in the conversion of lactate to acetate in *Lact. plantarum* (Goffin et al. 2006). Enzyme kinetic data from literature were used for construction of a detailed glycolytic model of *L. lactis* during glucose run-out experiments (Hoefnagel et al. 2002).

### 1.2.2. Control of the Glycolytic Flux

Despite intensive research and a wealth of metabolic information for *L. lactis*, the mechanisms controlling glycolytic flux in this and other LAB species remain unclear. This topic has been extensively discussed in excellent recent reviews (Cocaign-Bousquet et al. 2002; Neves et al. 2005; Kowalczyk and Bardowski 2007).

Several enzymes have been found to participate in the control of catabolic flux in *L. lactis*; however, it should be stressed that their action can be strain- and/or condition-dependent. During homolactic fermentation in *L. lactis* subsp. *lactis* NCDO 2118, the control has been attributed to glyceraldehyde-phosphate dehydrogenase (GAPDH), which is modulated by the NADH/NAD<sup>+</sup> ratio (Even et al. 1999). According to other results the key glycolytic enzymes phosphofructokinase (PFK), pyruvate



kinase (PK), and LDH or GAPDH exert, at normal activity levels, virtually no control on the glycolytic flux in *L. lactis* (Andersen et al. 2001; Koebmann et al. 2005). However, at reduced activities both PFK and PK were found to exert a strong positive control on glycolysis (Koebmann et al. 2005). Depending on culture conditions (e.g., oxygen and/or glucose concentration), the overall flux control seems to be distributed over many glycolytic steps and may also reside outside of the pathway (such as ATP-consuming reactions and sugar transport). Furthermore, the end-product demand might also be essential in flux control (Koebmann et al. 2002a, 2002b; Papagianni et al. 2007).

*Shift to mixed-acid fermentation.* Despite extensive studies the exact mechanisms underlying shift in the mode of fermentation remain elusive. Originally, the regulation of the switch was mainly attributed to the modulation of the intracellular concentration of fructose-1,6-bisphosphate (FBP), which allosterically activates both LDH and PK, and of glyceraldehyde-3-phosphate (GAP) and dihydroxyacetone-phosphate (DHAP) inhibiting the PFL. Inorganic phosphate has been shown to be a severe inhibitor of LDH and PFL (for a review, Coccagn-Bousquet et al. 2002; Neves et al. 2005).

A metabolic model involving modulation of GAPDH and LDH activities via the NADH/NAD<sup>+</sup> ratio (redox state) in *L. lactis* subsp. *lactis* NCDO 2118 has been postulated as a main factor regulating the shift in metabolism (Even et al. 1999). However, in other strains such as *L. lactis* subsp. *cremoris* MG1363 and *L. lactis* subsp. *lactis* IL1403, this hypothesis has not been confirmed. In these strains the glycolytic flux is not controlled by GAPDH (Even et al. 2001; Solem et al. 2003). Another study revealed that PK exhibits strong positive control on formate and acetate production and thus plays a role in the metabolic shift from homolactic to mixed-acid fermentation (Koebmann et al. 2005). In *L. lactis* subs. *lactis* IL1403 a high transcriptional level of the genes coding for PFL and alcohol dehydrogenase (ADH) was noted, while the activity of the enzymes was low, suggesting a post-transcriptional control (Even et al. 2001). Moreover, it has been demon-

strated that LDH may be co-regulated by at least two mechanisms (the FBP/inorganic phosphate [Pi] pool and the NADH/NAD<sup>+</sup> ratio), and the manner of regulation is strain-dependent (van Niel et al. 2004). Several studies suggest that in addition to an allosteric control, pyruvate metabolism may further be controlled at the transcription level of central catabolic pathways (Melchiorson et al. 2000; Even et al. 2001; Palmfeldt et al. 2004). Apart from the biochemical shift, regulation by allosteric changes of enzymes, metabolite levels, transcript, and protein levels, pyruvate metabolism is influenced by environmental factors such as oxygen, temperature, and pH (for a review, Neves et al. 2005; Kowalczyk and Bardowski 2007).

### 1.2.3. Heterofermentation

Heterofermentative LAB such as *Leuconostoc*, *Oenococcus*, and certain *Lactobacillus* species ferment sugars generally by the phosphoketolase pathway (PKP). Fermentation of pentoses (xylose, ribose) leads to the formation of pyruvate and acetyl-P and their subsequent conversion to lactate and acetate, respectively. Hexoses (glucose, fructose, mannose) in these bacteria can be converted to lactate, CO<sub>2</sub>, and ethanol (Fig. 1.1C). CO<sub>2</sub> is a product of 6-P-gluconate degradation, which occurs during conversion of hexoses to pentoses. The specific enzyme of the heterofermentative pathway, D-xylulose-5P phosphoketolase, catalyzes the conversion of xylulose-5P to GAP and acetyl-P. The first metabolite, GAP, enters the EMP pathway leading to the production of lactate, whereas the second metabolite, acetyl-P, is converted into ethanol. The low activity of the ethanol pathway in the reoxidation of the NAD(P)H due to a low acetaldehyde dehydrogenase activity limits heterofermentative growth on glucose (Maicas et al. 2002). Heterofermentative LAB bypass the limiting ethanol pathway by using alternative pathways for NAD(P)H reoxidation. Much higher growth rate on hexoses is observed when O<sub>2</sub>, pyruvate, citrate, or fructose is used as external electron acceptors (Zaunmüller et al. 2006; Arsköld et al. 2008). A part of the acetyl-P is then converted to acetate instead of

ethanol, making the PKP as efficient as the EMP pathway.

Recently, the simultaneous use of PKP and EMP pathways was reported in *Oenococcus oeni* and *Lactobacillus reuteri* ATCC 55730 (Richter et al. 2003; Arsköld et al. 2008). The use of two different glycolytic pathways has so far only been genetically supported in homofermentative lactobacilli, including *Lact. plantarum* (Pieterse et al. 2005), and *Lactobacillus salivarius* (Claesson et al. 2006). In both, EMP pathway and PKP, the growth phase-dependent expression of glycolytic pathways was demonstrated. Higher expression levels of the catabolic enzymes, such as PK, phosphoglycerate kinase, and GAPDH, during initiation of growth and log phase were reported in *L. lactis* subsp. *lactis* (Larsen et al. 2006). Similarly, proteins preferentially expressed in the early-exponential phase in *Lact. plantarum* were also associated with EMP and PKP pathways (Koistinen et al. 2007). This fact could be expected due to the positive effect of these enzymes on sugar consumption and biomass increase.

#### 1.2.4. Control of Sugar Metabolism in LAB

*The pivotal role of the HPr protein.* HPr is a part of the phosphoenolpyruvate (PEP)-PTS system, and in low-GC Gram (+) bacteria seems to be involved in different activities depending on its phosphorylation state. PEP-dependent phosphorylation of HPr by Enzyme I yields His-P-HPr, which serves as a phosphoryl carrier within the PTS phosphorylation cascade during transport of carbohydrates. However, His-P-HPr phosphorylates not only Enzyme II (EIIs) but also the key regulators of carbohydrate metabolism. For example, it phosphorylates histidyl residues in non-PTS proteins such as glycerol kinase, antiterminators, transcriptional activators, and non-PTS transporters. As a result of the metabolism of PTS substrates, the concerted action of FBP and an ATP-dependent protein kinase/phosphorylase (HprK/P) generates Ser-P-HPr. This form of HPr plays a crucial role in an inducer-exclusion mechanism and, together with the catabolic control protein A (CcpA), in carbon catabolite repression (CCR; for a review see Deutscher et al. 2006).

#### *Characteristics of CcpA-dependent regulation.*

The subject of CcpA-dependent regulation has been extensively described and discussed by several authors (Titgemeyer and Hillen 2002; Deutscher et al. 2006; Kowalczyk and Bardowski 2007). The CcpA protein from *L. lactis* IL1403 was successfully crystallized showing a potential cavity for a corepressor (Loll et al. 2007). The CcpA protein, together with its corepressor Ser-P-HPr, binds to 14 nucleotide cis-acting DNA target sites, called catabolite responsive elements (*cre*). Depending on the position of the *cre* sequence, CcpA can act as an activator or a repressor promoting carbon catabolite activation (CCA) or repression (CCR), respectively. *Cre* sites can either be located within the promoter, overlap the transcription start site, or lay between the transcription start site and the initiation codon or within the 5' part of catabolite-regulated genes. Binding of CcpA to *cre* sites overlapping the promoter region activates or represses the initiation of transcription, while binding to a *cre* situated downstream of the promoter leads to abortion of transcription. Most transcription units subjected to CCA contain *cre* sites in front of their promoter region. In the *L. lactis* IL1403 genome thousands of putative *cre* sequences have been detected (Andersson et al. 2005), although a majority may have no biological significance because of its location. Gene expression analyses using DNA microarrays have shown that CcpA regulates an impressive number of genes involved in carbohydrate transport and metabolism in *L. lactis* MG1363 (Zomer et al. 2007). Several genes containing functional *cre* sites, which are subjected to regulation by the Ser-P-HPr/CcpA complex have been recently described in a number of LAB (Titgemeyer and Hillen 2002; Deutscher et al. 2006; Francke et al. 2008). In *L. lactis* strains, CcpA has been shown to repress different  $\beta$ -glucosides, fructose, galactose, or lactose assimilation genes, but it activates the *las* operon (*pfk*, *pyk*, and *ldh* genes; Aleksandrak-Piekarczyk et al. 2005; Barriere et al. 2005; Kowalczyk et al. 2008). Interestingly, in *L. lactis* CcpA coordinates the metabolic switch from fermentative to aerobic growth, thus preventing oxidative damage (Gaudu et al. 2003). Non-PTS sugars can also trigger CcpA-

dependent carbon regulation, as is the case of lactose in *Strep. thermophilus* (van den Bogaard et al. 2000). In the genus *Lactobacillus* CcpA has been demonstrated to repress several sugar catabolic genes (Monedero et al. 1997; Chaillou et al. 2001; Muscariello et al. 2001; Marasco et al. 2002). Moreover, in *Lact. casei* and *Lactobacillus pentosus* CcpA has been shown to regulate glycolytic genes (*pfk* and *pyk*) and the central metabolic PKP, respectively (Posthuma et al. 2002; Viana et al. 2005b). In some LAB *ccpA* is preceded by a *cre*, and its expression is subjected to autoregulation (Morel et al. 2001; Zomer et al. 2007).

**Secondary regulators.** In addition to global regulators such as CcpA, carbon catabolism might also be controlled by specific, local regulators belonging to different protein families such as LacI, LysR, AraC, GntR, DeoR, or BglG, which are widely distributed among LAB. Regulators from these families have been proven to control genes for  $\alpha$ -galactosides, fructose, lactose, maltose, salicine, sorbose, and xylose assimilation in lactococci (Boucher et al. 2003; Kowalczyk and Bardowski 2007), *Strep. thermophilus* (Vaughan et al. 2001), or lactobacilli (Yebra et al. 2000; Fortina et al. 2003). For *L. lactis* and *Lact. plantarum* it has been shown that genes encoding secondary regulators are located in direct proximity and in a divergent transcriptional direction to the sugar uptake operon. As is the case for *ccpA*, most secondary regulators seem to undergo autoregulation (Francke et al. 2008).

**Control by factors containing PTS-regulated domains (PRD).** These regulators can activate the transcription of certain genes in the presence of the inducer by dephosphorylation of a PRD (though their activity can also be modulated by His-P-HPr phosphorylation of conserved histidine residues in a second PRD) in such a way that in the presence of rapidly metabolizable carbon sources, the regulators would be inactive (antitermination). The action of all known antiterminators depends on binding to a ribonucleic antiterminator sequence, resulting in the unwinding of a neighboring terminator structure in their respective mRNA (Yang et al. 2002). This

mechanism has been considered an alternative form of CCR mediated by HPr (Deutscher et al. 2006; Kowalczyk and Bardowski 2007). Among LAB, there are only two antiterminator proteins described so far: BglR from *L. lactis* (Bardowski et al. 1994) and LacT from *Lact. casei* (Gosalbes et al. 1999), both belonging to the BglG family. More recently, the identification of an operon controlled by a LevR-like regulator (strongly resembling the best studied PRD-containing transcription activator LevR of *Bacillus subtilis*) has been reported in *Lact. casei*, which encodes proteins of a mannose PTS (Maze et al. 2004).

**Inducer exclusion/expulsion.** These two protein-mediated mechanisms of carbon regulation supplement the above-mentioned mechanisms of transcriptional control (Barriere et al. 2005; Monedero et al. 2008). Based on *in vivo* analysis, Ser-P-Hpr has been proposed to drive the exclusion of the inducer in *Lact. casei*, *L. lactis*, and *Lactobacillus brevis*, but not the expulsion of the inducer (Monedero et al. 2008).

### 1.2.5. Exopolysaccharides (EPS)

LAB are able to direct some part of the sugar pool toward biosynthesis of EPS. These long-chain saccharides can be loosely attached to the cell surface forming some kind of capsules or are secreted to the environment. EPS represent molecules with different structures, sizes, and sugar composition (reviewed by Laws et al. 2001; Broadbent et al. 2003; Ruas-Madiedo et al. 2008). They are classified into two groups: homopolysaccharides consisting of one type of monosaccharide ( $\alpha$ -D-glucans,  $\beta$ -D-glucans, fructans, and others represented by polygalactan) and heteropolysaccharides composed of different types of monosaccharides, mainly D-glucose, D-galactose, L-rhamnose, and their derivatives. In the last few years several works presented a diversity of biopolymers produced by cereal-associated and intestinal LAB (Tiekink et al. 2003; van der Meulen et al. 2007), *Strep. thermophilus* (Vaningelgem et al. 2004) or other LAB strains isolated from dairy products (Mozzi et al. 2006; van

der Meulen et al. 2007). During these studies the production of glucan by *L. lactis*, an EPS-containing (N-acetyl) glucosamine by *Strep. thermophilus* (Vaningelgem et al. 2004), a polygalactan synthesized by two strains of *Lactobacillus delbrueckii* subsp. *bulgaricus* (Mozzi et al. 2006), and a heteropolysaccharide by *Lactobacillus curvatus* (van der Meulen et al. 2007), has been reported for the first time. Pioneering research describing the detection of EPS-producing strains in the human intestinal ecosystem showed that 17% of *Lactobacillus* and *Bifidobacterium* strains could be EPS producers (Ruas-Madiedo et al. 2007).

**Biosynthesis of EPS.** Homopolysaccharides are synthesized extracellularly by highly specific glycosyltransferase enzymes, glucan or fructan sucrases (van Hijum et al. 2006). This process requires sucrose as a specific substrate and the energy that comes from its hydrolysis. Recently, a negative effect of glycosyltransferases on growth on sucrose of some *Lact. reuteri* strains has been observed (Schwab et al. 2007). EPS belonging to heteropolysaccharides are synthesized from glucose, galactose, or other monosaccharides by a combined action of different types of glycosyltransferase enzymes. Four major consecutive steps of EPS biosynthesis in LAB involve sugar transport into the cytoplasm, synthesis of sugar-1P, polymerization of repeating unit precursors, and finally EPS export outside the cell (reviewed by Laws et al. 2001; Welman and Maddox 2003).

**Regulation of EPS production.** It is postulated that regulation of EPS production can be possible at each of the different steps during biosynthesis. It has been observed that the overexpression of the fructose biphosphate (*fbp*) gene led to an increase of the EPS yield in *L. lactis* cells growing on fructose. The role of enzymes, such as glucokinase, PFK, and PGM controlling the carbon distribution between catabolic (glycolysis) and anabolic reactions (EPS precursor biosynthesis) has recently been studied in *L. lactis* (Boels et al. 2003). It has been shown that overproduction of PGM resulted in increased levels

of both UDP-glucose and UDP-galactose; however, did not significantly affect EPS production. In *L. lactis*, EPS production is controlled by a phosphoregulatory system and EPS biosynthesis correlates with an unphosphorylated protein (EpsB) (Nierop Groot and Kleerebezem 2007).

### 1.2.6. The Post-genomic Era of Sugar Metabolism and Regulation in LAB

At the time of writing complete genome sequences have been presented for more than 20 fermentative and commensal LAB species from the order of *Lactobacillales* and more genome projects are ongoing (Mayo et al. 2008). Most genes involved in sugar metabolism and energy conversion are uniformly represented among LAB species with completed genome sequences (Makarova et al. 2006). Moreover, it seems that genes encoding enzymes involved in glycolysis, pyruvate metabolism, and/or regulators such as CcpA, HPr, HprK/P, and EI are highly expressed in *L. lactis* and *Lactobacillus acidophilus* (Guillot et al. 2003; Karlin et al. 2004; Barrangou et al. 2006).

Genome analyses have also shown that lactobacilli, streptococci, and lactococci, probably due to the diversity of environments they inhabit, possess wide saccharolytic abilities. The ability to ferment many different kinds of carbohydrates is a specific characteristic of plant-associated strains (Nomura et al. 2006). Analysis of the genome of different *Lact. plantarum* strains has revealed the existence of many genes involved in sugar transport and metabolism located in the so-called "lifestyle adaptation island," a 213 kb region of high plasticity (Kleerebezem et al. 2003; Molenaar et al. 2005). Furthermore, unique genes and clusters for xylan, glucan, and fructan/mannan breakdown have been identified in the genome of nondairy *L. lactis* subsp. *lactis* strains isolated from plant material (Siezen et al. 2008). On the other hand, in milk-specialized bacteria, for example *Strep. thermophilus*, the capacity to degrade starch or glucans has been shown to be severely reduced (Salzano et al. 2007).

### 1.2.7. Practical Aspects of Sugar Catabolism

The ability of LAB to ferment sugars has been widely utilized in various food production processes. Recently, great attention has been directed to optimization of lactate production from natural substrates like starch or cellulose derived from waste products (for a review see, John et al. 2007; Reddy et al. 2008). Probiotic effects related to lactose catabolism have been the subject of several studies. Of particular interest is the selection of potential probiotic bacteria exhibiting high lactose hydrolysis (Drouault et al. 2002; Honda et al. 2007). The elucidation of the metabolic adaptation of bacteria to lactose in the digestive tract is also important (Roy et al. 2008). Some probiotic LAB can utilize prebiotic compounds such as non-digestible fructooligosaccharides (FOS) or inulin-type fructans, which stimulate the growth of beneficial commensals in the gastrointestinal tract. Lactic acid has been shown to be the main metabolic end-product of oligofructose-enriched inulin (OEI) fermentation by *Lactobacillus paracasei* subsp. *paracasei* 8700:2, although significant amounts of acetic acid, formic acid, and ethanol were also produced when long-chain inulin or OEI was used as the sole energy source (Makras et al. 2005). *Lact. acidophilus* 1195 hydrolyzes FOS by a fructosidase, which renders fructose and sucrose; the latter sugar is subsequently hydrolyzed into fructose and glucose-1P by a sucrose phosphorylase (Barrangou et al. 2003; Goh et al. 2006). In *Lact. plantarum* a sucrose PTS, a  $\beta$ -fructofuranosidase, and a fructokinase were found to be involved in the degradation of short-chain FOS (Saulnier et al. 2007). As already mentioned, LAB can direct part of the sugar pool toward biosynthesis of EPS. These microbial polysaccharides have been used in the food industry as emulsifiers, thickeners, viscosifiers, and stabilizers. Important studies from a practical point of view concern the kinetics of EPS formation and factors affecting EPS production, such as nutrients (Velasco et al. 2006), type of carbon and nitrogen sources (Korakli et al. 2003; Sánchez et al. 2006; Velasco et al. 2006), tempera-

ture, and pH (Zisu and Shah 2003; Sánchez et al. 2006).

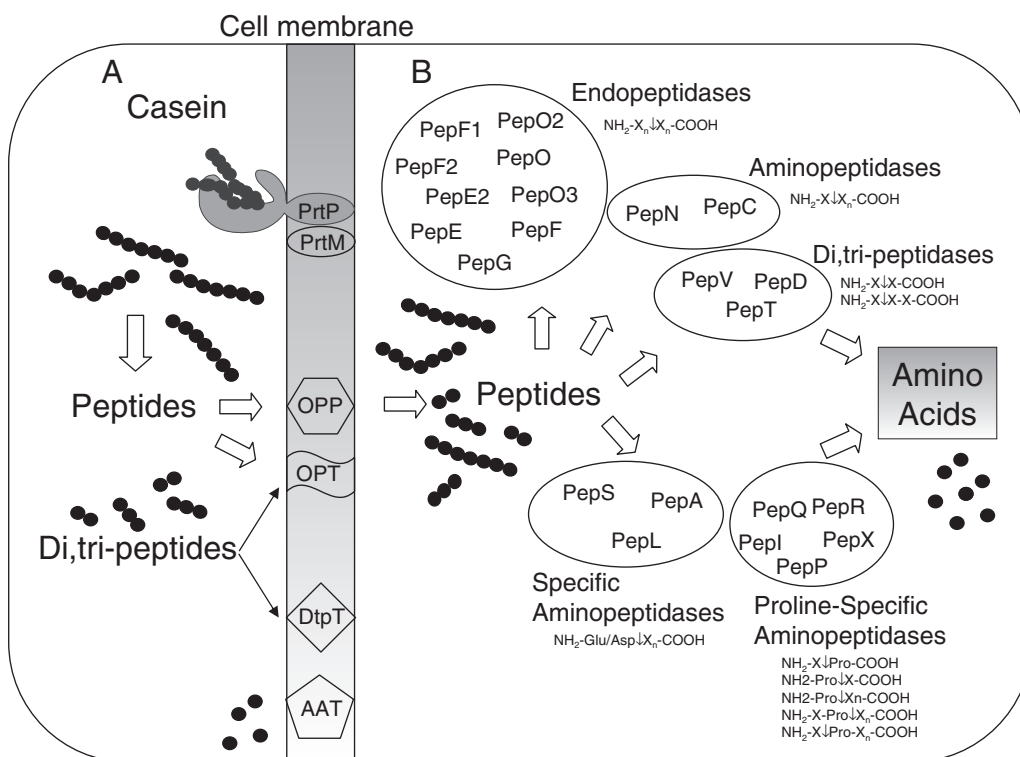
Finally, it is worth mentioning the protective role of sugars in the preservation and storage of LAB species. Sugars such as trehalose, sorbitol, mannitol, sucrose, lactose, and mannose increased the viability of the cells during drying and freezing processes (reviewed by Santivarangkna et al. 2008). In addition, the mechanism involved in the protective effect of glucose on *Lactobacillus rhamnosus* GG during gastric transit has been shown to rely on an increased activity of the  $F_0F_1$ -ATPase enabling proton exclusion (Corcoran et al. 2005).

## 1.3. Proteolysis and Amino Acid Catabolism

It has been well established that LAB species are auxotrophs for a variable number of amino acids, depending both on the species and on the strain. Therefore, for proper growth on most systems LAB depend on a fully active proteolytic system to meet their amino acid requirements. The proteolytic components of dairy LAB are among the best characterized to date, not only as a consequence of their impact on the physiology of LAB, but also because of their involvement in the development of texture and flavor in dairy products (for recent reviews see, Smit et al. 2005; Savijoki et al. 2006). The recent analysis of LAB genomes has further contributed to a complete characterization of the proteolytic systems in the sequenced species (Mayo et al. 2008). The genome of *Lact. plantarum* is the only LAB which encodes enzymes for the biosynthesis of all amino acids, except for leucine, isoleucine, and valine (Kleerebezem et al. 2003). In contrast, *Lact. acidophilus* is likely to be auxotrophic for 14 amino acids (Altermann et al. 2005), and *Lactobacillus johnsonii* appears incapable of de novo synthesis of most, if not all, amino acids (Pridmore et al. 2004).

The structural components of the proteolytic systems can be divided into three groups on the basis of their function: (1) proteinases breaking proteins into peptides; (2) transport systems that





**Figure 1.2.** Diagram of the proteolytic systems of lactic acid bacteria. Location and targets of the different components are indicated. The pentagon at the bottom of the cell membrane marked as AAT refers to the different types (about 14) of amino acid transporters. Action and specificity of peptidases over a schematic peptide is indicated by arrows.

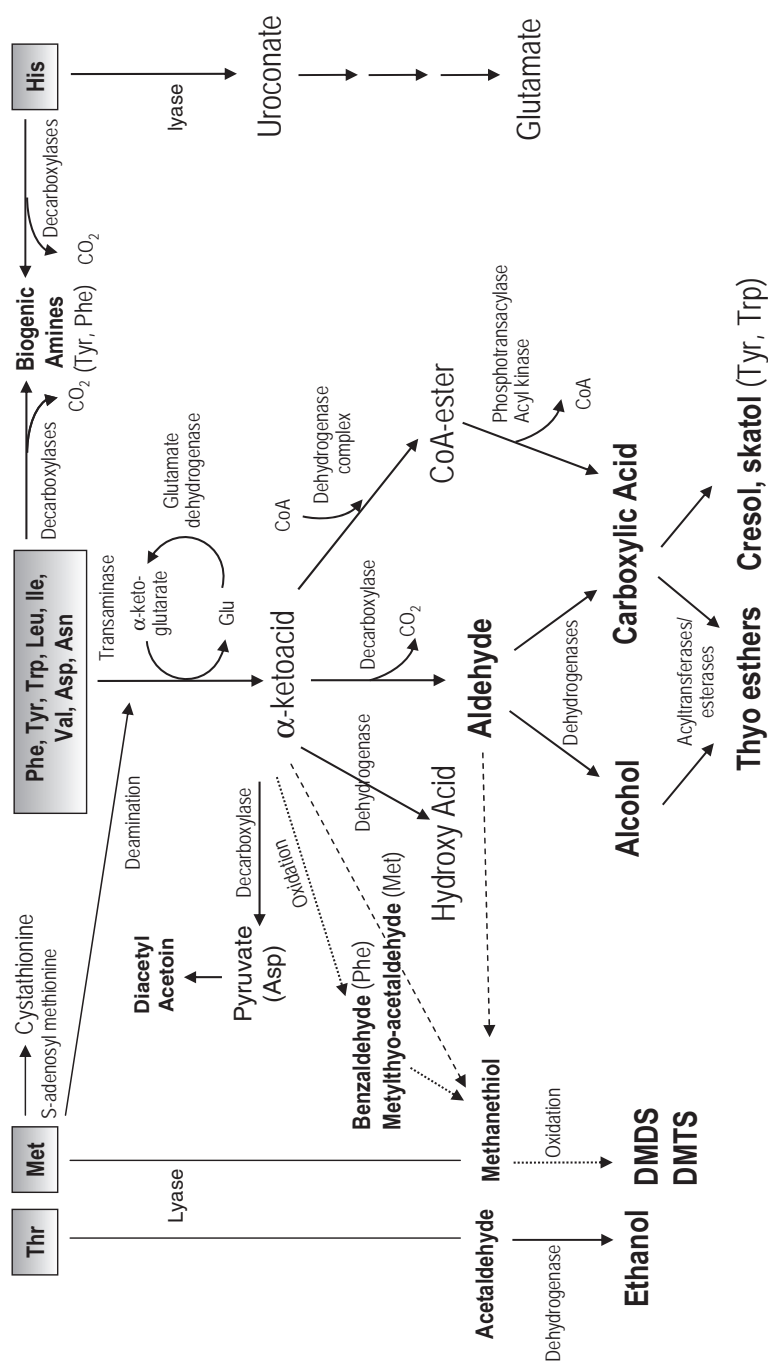
translocate the breakdown products across the cytoplasm membrane; and (3) peptidases that degrade peptides into free amino acids (Fig. 1.2). These amino acids will be further degraded by strain-dependent metabolic pathways to generate the actual volatile compounds responsible for the aroma profile of fermented products (Fig. 1.3).

### 1.3.1. Proteinases

A single cell wall-bound extracellular proteinase (CEP) is generally considered to be responsible for the initial breakdown of caseins. Gene deletion studies have shown that LAB strains cannot grow in milk in absence of a functional CEP. This enzyme is a serine protease belonging to the subtilisin family. Five different types of this enzyme have been characterized from LAB: PrtP from *L. lactis*

and *Lact. paracasei*, PrtH from *Lactobacillus helveticus*, PrtR from *Lact. rhamnosus*, PrtS from *Strep. thermophilus*, and PrtB from *Lact. delbrueckii* subsp. *bulgaricus* (*Lact. bulgaricus*). All enzymes are synthesized as pre-pro-proteins, of approximately 2000 residues, consisting of several functional domains (Siezen 1999).

In general, LAB species possess only one CEP each; however, the presence of two enzymes has been described in some lactobacilli (Pederson et al. 1999). A membrane-bound lipoprotein, PrtM, has been found to be essential for the autocatalytic activation of the proteinase in *L. lactis* and *Lact. paracasei*. Moreover, *prtP* and *prtM* genes are divergently transcribed (Haandrikman et al. 1991). In contrast, the maturation gene has not been identified in the CEP-flanking regions of *Lact. helveticus*, *Lact. bulgaricus*, and *Strep. thermophilus* (Fernández-Esplá



**Figure 1.3.** Catabolic pathways of the principal amino acids involved in the production of aroma compounds. Continuous lines show enzymatic reactions catalyzed by the enzymes indicated. Dotted and broken lines show spontaneous chemical reactions and poorly defined pathways, respectively. The most important compounds formed are shown in bold.

et al. 2000; Germond et al. 2003). However, recent microarray analysis has shown an increase expression of a homologous of *prtM* in *Lact. helveticus* CNRZ32 when growing in milk (Smeianov et al. 2007), suggesting that at least in this organism a PrtM-like protein may play a role in CEP maturation. Expression of *prtP* in *L. lactis* is repressed in the presence of rich nitrogen sources such as casein hydrolysates, casamino acids, and specific dipeptides. The expression is under the control of the general regulator CodY, which senses the internal branched-chain amino acid (BCAA) pool (Guédon et al. 2001). Regulation of the proteolytic system in lactobacilli is much less studied, and, as in *L. lactis*, rich nitrogen sources represses proteinase expression (Hebert et al. 2000; Pastar et al. 2003). Some species of LAB do not exhibit CEP-like activity, such as *Lact. plantarum* (Kleerebezem et al. 2003) and other lactobacilli species from sourdough (Vermeulen et al. 2005). This suggests that caseinolytic proteinases may function as an adaptative factor, which has been acquired by LAB of dairy and intestinal origin.

Intracellular proteinases involved in regulatory networks and stress responses of LAB, required for normal growth under different stress conditions, such as FtsH, HtrA, and Clp-proteases (Poquet et al. 2000; Foucaud-Scheunemann and Poquet 2003; Savijoki et al. 2003) might also play a role during maturation of fermented products.

### 1.3.2. Peptide and Amino Acid Uptake Systems

The second step in protein degradation includes the transport of di-, tri-, and oligo-peptides into the cell by the action of different peptide uptake systems (Fig. 1.2). Three functional peptide transport systems have been described in *L. lactis*: DtpT, Opp, and Opt (previously referred to as Dpp). DtpT is a proton motive force-dependent transporter whose specificity is limited to di- and tripeptides (Kunji et al. 1993). This peptide uptake system is also present in *Lact. helveticus*, *Lactobacillus sanfranciscensis*, and *Strep. thermophilus*. Opp and Opt are transport-

ers belonging to the ABC transporter superfamily, which transport di-, tripeptides, and oligopeptides (Detmers et al. 1998). They have been identified in different species of LAB, such as *L. lactis*, *Lact. bulgaricus*, *Lact. helveticus*, *Lact. plantarum*, *Lact. sanfranciscensis*, and *Leuconostoc mesenteroides*. Opp was characterized first and is composed of five proteins: an oligopeptide-binding protein (OppA), two integral membrane proteins (OppB and OppC), and two nucleotide-binding proteins (OppD and OppF; Tynkkynen et al. 1993). Opt differs from Opp by its genetic organization and peptide uptake specificity. The Opt system contains two different peptide-binding proteins. Initially, the Opt system was only related with the transport of di- and tri-hydrophobic peptides. However, analysis of Opp-deficient *L. lactis* strains revealed the implication of Opt in the transport of oligopeptides (Foucaud et al. 1995). Differences in specificity between the Opp and Opt are likely to be responsible for the previously observed variability in peptide uptake within different strains of *Lactococcus* (Charbonnel et al. 2003). Specificity has been shown to be imposed by the (oligo)peptide-binding proteins (Doeven et al. 2005). A second set of Opp-transporter encoding genes, named *opp2*, was found in *L. lactis* MG1363, although this latter Opp system is either not expressed or not functional (Doeven et al. 2005). All these three systems (Opp, Opt, and DtpT) have been found to be functional in only a minority of *L. lactis* strains (such as MG1363, SK11, and Wg2; Doeven et al. 2005). The Opp from IL1403 and SKM6 lacks the OppA component. Thus, most likely, uptake of oligopeptides in these strains seems to be driven by Opt.

In *Strep. thermophilus*, the oligopeptide transport system presents some differences from that of *Lactococcus*. It has been called Ami, since it shows the highest identity with the Ami system of *Streptococcus pneumoniae* (Garault et al. 2002). However, the specificity of the *Strep. thermophilus* oligopeptide uptake is broader than those of streptococci and similar to that specified by OppA from *L. lactis* (Garault et al. 2002). The Ami transporter is composed of three oligopeptide-binding proteins (AmiA1, AmiA2, and AmiA3), two-membrane

proteins (AmiC, AmiD), and two ATPases, which provide energy to the system (AmiE, AmiF).

A variable number of genes encoding putative amino acid permeases and transporters of several classes have been identified in the genome of LAB species (Makarova et al. 2006). However, specificity of most transporters is not yet known.

### 1.3.3. Peptidases

Inside the cells, peptides are degraded by the concerted action of an array of peptidases with distinct but overlapping specificities (Kunji et al. 1996a). Peptidases can be divided into two types: endopeptidases, which hydrolyze internal peptide bonds, and exopeptidases, which remove amino acids from an extreme of the peptide chain. In LAB a majority of the exopeptidases are aminopeptidases and their specificity depends on the peptide length and the nature of the N-terminal amino acid residue (Kunji et al. 1996b; Fig. 1.2).

**Endopeptidases.** PepO was the first endopeptidase characterized from a LAB species. It is a 70-kDa monomeric metalloprotease capable of hydrolyzing peptides from 5 up to 35 residues, but unable to hydrolyze intact caseins. The inspection of sequenced genomes has shown that all LAB encode putative PepO homologs. *L. lactis* MG1363 encodes a second endopeptidase with properties similar to PepO, named PepO2. Additionally, the analysis of the *Lact. helveticus* CNRZ32 genome revealed a third PepO endopeptidase, PepO3, which is a functional paralog of PepO2, and both endopeptidases (PepO2 and PepO3) could play a key role in the *Lact. helveticus* CNRZ32 ability to reduce bitterness in cheese (Savijoki et al. 2006). Duplication of peptidases would enhance the ability of *Lactobacillus* species to exploit protein-rich environments (Makarova et al. 2006). A second endopeptidase with a different specificity from that of PepO has been designated as PepF1. It is a 70-kDa monomeric metallopeptidase, which hydrolyzes peptides containing between 7 and 17 amino acids with a rather broad specificity. In *L. lactis* subsp. *cremoris* NCDO763 two copies of this gene have

been found, one, *pepF1*, is plasmid encoded, whereas a second copy, *pepF2* (80% identity), is located in the chromosome. PepF has also been identified in other LAB genomes, such as *Lact. helveticus*, *Lact. brevis*, *Strep. thermophilus*, *O. oeni*, *Leuc. mesenteroides*, *Lactobacillus gasseri*, and *Lact. delbrueckii*. Other endopeptidase genes have further been identified in *Lact. delbrueckii* (PepG; van de Guchte et al. 2006) and *Lact. helveticus* (PepE and PepE2; Callanan et al. 2008).

**Aminopeptidases.** The aminopeptidases identified in LAB can be divided into three groups: aminopeptidases with broad specificity, specific aminopeptidases for acidic or basic amino acids, and those specific for hydrophobic or aromatic residues. Aminopeptidases with broad specificity, PepC and PepN, have been identified in *Lactococcus*, *Lactobacillus*, and *Strep. thermophilus*. PepC belongs to the C1 family of cysteine peptidases and has a significant activity on basic, acidic, hydrophobic/uncharged, and aromatic residues. PepN presents a higher activity on basic residues followed by hydrophobic/uncharged residues and, in general, low or undetectable activity on Asp, Glu, and Gly (Savijoki et al. 2006). Aminopeptidases acting on acidic residues, PepA, also referred to as glutamyl aminopeptidases, have been identified in *L. lactis*, *Strep. thermophilus*, and *Lact. gasseri* genomes. This enzyme belongs to M42 family of metallopeptidases. Since bitter peptides are mainly formed by hydrophobic and aromatic residues, an aminopeptidase specific for aromatic amino acids could have an important role in the development of flavor in dairy products. An enzyme with this activity, PepS, has been identified in *Pediococcus pentosaceus*, *Strep. thermophilus*, *Leuc. mesenteroides*, *Lact. casei*, and *Lact. sakei*. PepL, an aminopeptidase that has only been identified in *Lact. delbrueckii*, displays high specificity for Leu and Ala residues (Savijoki et al. 2006).

**Dipeptidases.** Several dipeptidases have been described in LAB with a broad specificity (PepD and PepV) and with specificity to peptides containing proline (PepQ and PepR; see below). PepD has

only been characterized from *Lact. helveticus*. It has an activity similar to that reported for PepV but it is unable to hydrolyse Val-X and Ile-X dipeptides (Savijoki et al. 2006). Analysis of available LAB genomes has lead to the identification of encoding proteins similar to PepV and PepD dipeptidases in other *Lactobacillus* species as well as in *L. lactis* and *Strep. thermophilus*.

**Tripeptidases.** These enzymes hydrolyze tripeptides with a wide range of amino acid composition although they do not hydrolyze tripeptides containing Pro in the middle position. The tripeptidase PepT has been identified in *L. lactis*, *Strep. thermophilus*, and *Lact. helveticus*. Other tripeptidases have been purified from other LAB (*Lact. sakei*, *Lact. delbrueckii*, *Ped. pentosaceus*) and, like PepT, they are all metallopeptidases having a high activity on tripeptides composed of hydrophobic/uncharged and aromatic residues.

**Proline-specific peptidases.** This group of peptidases includes aminopeptidases and dipeptidases (PepQ, PepR, PepI, PepP, and PepX), which are specific for Pro residues. PepQ is a prolidase enzyme specific for X-Pro dipeptides although in some cases is able to hydrolyze other dipeptides. This enzyme has been characterized from different *Lactobacillus* and *Lactococcus* species. PepR is a dipeptidase identified only in some *Lactobacillus* species with prolidase activity, specific for Pro-X dipeptides, which has a limited activity in larger peptides. PepI releases Pro from the N-terminus of tri-peptides, but not from tetra- and penta-peptides. PepP is an aminopeptidase releasing the N-terminal amino acid from peptides with the X-Pro-Pro-(Y)<sub>n</sub> composition. This enzyme has been characterized from *L. lactis* and has high activity on peptides ranging from three to nine residues, with the highest rate of hydrolysis on pentapeptides. PepX releases X-Pro dipeptides from substrates containing three to seven amino acid residues. This enzyme is widely distributed among dairy LAB, and it surely is the most thoroughly studied Pro-specific peptidase. Prolidase enzymes such as PepQ can subsequently hydrolyze the dipeptides released by PepX (Savijoki et al. 2006).

#### 1.3.4. Regulation of the Proteolytic System

The proteolytic system of *L. lactis* has been shown to respond to changes in nitrogen availability. It is now clear that the transcriptional regulator CodY negatively regulates the expression of several components of the proteolytic system in this species by binding to their cognate promoter regions (den Hengst et al. 2005). Repression is modulated by the intracellular pool of BCAAs (Guédon et al. 2001). However, not all proteolytic enzymes respond to this regulation. Expression of *pepP* has been demonstrated to respond to catabolite repression and is controlled by CcpA (Guédon et al. 2001).

The regulatory mechanisms are much less studied in *Lactobacillus*. Peptide concentration in the growth medium has been shown to control PrtH and PrtR biosynthesis in *Lact. helveticus* and in *Lact. rhamnosus*, respectively. A recent systematic study on *Lact. acidophilus* using expression profiling with whole genome microarrays has revealed that a two-component regulatory system acts as a pleiotropic regulator in controlling the expression of at least 80 genes, including major components of the proteolytic system (Azcárate-Peril et al. 2005). To date, only one regulatory protein directly controlling the expression of proteolytic genes has been identified in *Lactobacillus*. In *Lact. delbrueckii* subsp. *lactis* and *Lact. bulgaricus*, PepR1, a CcpA-like regulator, has been demonstrated to bind to the promoter regions of *pepQ* and *pepX*, acting as a transcriptional activator. In this case, expression of PepQ depends on carbohydrate concentration and composition rather than on peptide concentration; this contrasts with the biosynthesis of other components of the proteolytic system in *Lact. bulgaricus* (Morel et al. 2001).

#### 1.3.5. Physiological Role and Technological Aspects of Proteolysis

Analyses conducted on mutant strains of *L. lactis*, *Lact. helveticus*, *Lact. rhamnosus*, and *Strep. thermophilus* defective in components of the proteolytic system have shown that PrtP and Opp are crucial for growth in a medium with casein as the sole source



of amino acids (Christensen et al. 1999). Numerous LAB strains with mutations in single peptidase genes have also been examined for their ability to grow in milk; although minor changes in growth were observed, it can be concluded that none of the individual peptidases is essential for growth in milk because its activity is compensated by other peptidases (Kunji et al. 1996b). Proteolysis is considered one of the most important biochemical processes involved in the ripening of many fermented dairy products. It releases amino acids from caseins, which, in turn, are major precursors of specific flavor compounds: alcohols, aldehydes, acids, esters, and sulfur compounds. As mentioned before, the peptidolytic activity (PepN, PepX, PepO2, and PepO3) is also important in reducing the accumulation of hydrophobic peptides to reduce bitterness in cheese (Sridhar et al. 2005).

Besides the importance of proteolytic enzymes in the final organoleptical properties of fermented products, certain LAB strains are further known to be able to liberate bioactive peptides encrypted in proteins, which are thought to have a role in promoting health (Korhonen and Pihlanto-Leppälä 2002). To date, strains of LAB endowed with such properties include strains such as *Lact. helveticus* CP790, *Lact. rhamnosus* GG, *Lact. bulgaricus* SS1, and *L. lactis* subsp. *cremoris* FT4 (Gobetti et al. 2002; Muguerza et al. 2006; see Chapter 13 for more details). Several reports have indicated that bioactive peptides may also be produced *in vivo* by intestinal LAB after the intake of milk proteins (Meisel 2004). Production of such peptides at an industrial scale for use as dietary supplements is currently receiving increased interest (Meisel 2004).

### 1.3.6. Amino Acid Catabolism and Flavor Formation

The catabolism of amino acids has implications with regard to quality (formation of flavor) and safety (synthesis of biogenic amines) of fermented foods. Amino acid catabolism is further believed to have an important role in LAB physiology for obtaining energy in nutrient-limited conditions, participating in pH homeostasis. However, most pathways remain

only partially characterized in LAB. BCAAs (valine, isoleucine, and leucine), aromatic amino acids (tyrosine, tryptophane, and phenylalanine), sulfur-containing amino acids (methionine and cysteine), and threonine are the main amino acid sources for flavor (sweaty, sour, and sweet aroma; Fig. 1.3). Other amino acids can participate as well; for instance, the catabolism of aspartic acid has been recently shown to be involved in production of both acetoin and diacetyl (Le Bars and Yvon 2008).

**BCAAs.** BCAAs and sulfur-containing amino acids can be degraded by two distinct pathways: transamination and degradation (Fig. 1.3). Transamination can be catalyzed by two aminotransferases, BcaT and AraT (Yvon and Rijnen 2001), which convert amino acids into their corresponding  $\alpha$ -ketoacid. BcaT orthologs are present in all lactococcal and streptococcal strains while they are absent in a number of lactobacilli. The gene encoding AraT has been experimentally characterized in *L. lactis* and putative *araT* genes have been found in all LAB genomes except those of *Lact. sakei* and *Lact. brevis*. The *araT* gene is monocistronically transcribed and it is under the control of CodY. The transaminase reaction is commonly linked to the deamination of glutamate to  $\alpha$ -ketoglutarate, catalyzed by glutamate dehydrogenase (GDH). GDH activity varies widely among LAB, and most strains of *L. lactis* apparently lack this activity (Lapujade et al. 1998) while it is present in the majority of *Strep. thermophilus* strains (Helinck et al. 2004). In lactobacilli, GDH activity is also strain dependent (Kieronczyk et al. 2003). Inspection of available LAB genomes has revealed that GDH-encoding genes are present in some strains (e.g., *Lact. plantarum*, *Lact. sakei*, *Strep. thermophilus*) but absent in others (e.g., *L. lactis*, *Lact. casei*, *Lact. delbrueckii*).

The  $\alpha$ -ketoacids can be further converted to aldehydes, carboxylic acids, and hydroxyacids in three different routes: oxidative decarboxylation to carboxylic acids, decarboxylation to aldehydes, and reduction to hydroxyacids (Fig. 1.3).  $\alpha$ -Ketoacids can be decarboxylated to aldehydes by  $\alpha$ -ketoacid decarboxylases. In LAB this activity has been

reported in *L. lactis* var *maltigenes*, *Lact. casei*, and *Lact. delbrueckii* subsp. *lactis* (Helinck et al. 2004) and in some nondairy *L. lactis* strains (Smit et al. 2004). Recently, this enzyme has been characterized from two different *L. lactis* strains and shown to require thiamine pyrophosphate as a cofactor. The encoding gene (*kdcA*, also called *kivD*) shows homology with indole pyruvate decarboxylases (de la Plaza et al. 2004; Smit et al. 2005). Aldehydes produced in these reactions can then be reduced to alcohols by alcohol dehydrogenases or oxidized to carboxylic acids by aldehyde dehydrogenases (Yvon and Rijnen 2001).

Oxidative decarboxylation of BCAAs to carboxylic acids is uncommon in LAB. This reaction leads to the formation of carboxylic acids without transitory formation of aldehydes. In the first step, the  $\alpha$ -ketoacids are reductively decarboxylated to their corresponding acyl-CoA by  $\alpha$ -keto acid dehydrogenase (KADH), an enzyme complex composed by four subunits. Acyl-CoA is further converted to the corresponding carboxylic acid by a phosphotransacylase and acyl kinase (ACK; Fig. 1.3). Orthologous genes of components from the oxidative decarboxylation pathway (*ptb-buk-bkdDABC*) have only been found in the *Lact. casei* genome. Nevertheless, homologs of the *ptb* gene, *buk* gene, and *bkdDABC* genes were found encoded separately in different positions of the chromosome in various other LAB, for instance in *Strep. thermophilus*. This finding agrees well with the experiments of Helinck et al. (2004), who showed these enzyme activities in *Strep. thermophilus* strains. Caution is required, however, since the best homologs of KADH in many LAB are annotated as either pyruvate or acetoin dehydrogenase complex, and it is not clear whether these complexes have overlapping substrate specificity (Liu et al. 2008).

Keto acids can also be reduced to hydroxy acids by hydroxy acid dehydrogenase (HADH). Two stereospecific enzymes, D-HADH and L-HADH, are distinguished, which belong to the larger D-LDH and L-LDH protein families, respectively. L-HADH from *Weissella confusa* (formerly *Lactobacillus confusus*) has been characterized, and a D-HADH

encoding gene has been cloned from *Lact. casei*. Although there is no literature evidence that hydroxy acids can directly lead to flavor formation, the fact that precursors of hydroxy acids and some flavor compounds are shared imply that the activity of HADH could have a negative effect on flavor formation by shunting flavor precursors into off-flavors.

ADH and aldehyde dehydrogenase (ALDH) catalyze the conversion of aldehydes to alcohols and carboxylic acids, respectively. Most LAB genomes encode multiple ADH members, but only a single ADH/ALDH ortholog; these activities have never been studied in LAB species.

*Methionine, cysteine, and threonine.* Sulfur compounds produced from the catabolism of sulfur-containing amino acids are potent odorants in many fermented foods. For this reason Met and Cys catabolism has been deeply studied in the last years. The metabolism of sulfur-containing amino acids is complex, considering the existence of multiple alternative pathways/enzymes and a species and strain distribution of these activities. Met can be metabolized by (1) conversion to cystathionine through S-adenosyl methionine, thus linking Met and Cys pools; (2) deamination to  $\alpha$ -oxo- $\gamma$ -methylthiobutyrate by an aminotransferase reaction; and (3) simultaneous deamination and dethiomethylation to methanethiol by Met lyases (Fig. 1.3). The conversion of Met into methanethiol via lyase can be catalyzed by three PLP-dependent enzymes: cystathionine  $\beta$ -lyase (CBL), cystathionine  $\gamma$ -lyase (CGL), and Met  $\gamma$ -lyase (MGL). MGL is widely distributed among bacteria, but it has not been identified in LAB. CBL and CGL can use various sulfur-containing substrates including Met to produce methanethiol via  $\alpha$ - and  $\gamma$ -elimination. CBL- and CGL-encoding genes have been identified in many LAB genomes, but these lyases possibly play a minor role in Met degradation, at least in *L. lactis* (Fernández et al. 2000). Met can also be degraded via transamination (Fig. 1.3). The resulting product, 2-oxo-4-(methylthio) butyric acid (KMBA), can be subsequently transformed into methanethiol by an unknown pathway (Yvon and Rijnen 2001). In lac-



tobacilli and *Strep. thermophilus*, transamination by AraT and BcaT is the main pathway for Met degradation (Amárita et al. 2001). The resulting KMBA can be chemically converted to methylthioaldehyde, methanethiol, and dimethyl sulfide. Methanethiol can be auto-oxidized to dimethyl sulfide and dimethyl trisulfide (Fig. 1.3). However, KMBA can also be degraded to methanethiol and 2-hydroxyl-4-methylthiobutyric acid by an enzymatic pathway in some lactococcal strains. Decarboxylation of KMBA to methional has been reported in one strain of *L. lactis* although by unknown pathway (Amárita et al. 2002).

Very few data are available on Cys catabolism in LAB. Amino acid utilization studies have shown that most LAB can utilize Cys, although this ability is strain-dependent (Williams et al. 2001). In addition, it has been reported that CGL can degrade Cys and cystine (the latter being the best substrate) to ammonia, hydrogen sulfide, and pyruvate (Bruinenberg et al. 1997). This activity, also referred to as Cys desulfhydrase, has been reported in *Streptococcus anginosus* and other streptococci (Yoshida et al. 2003). Cys could also be utilized for Met biosynthesis via cystathionine, which might be subsequently degraded.

Threonine can be degraded to acetaldehyde (Fig. 1.3), a major flavor component of yoghurt. A Thr aldolase has been described as the key enzyme of the Thr catabolic pathway in LAB (Christensen et al. 1999). This enzyme catalyzes the splitting of Thr into acetaldehyde and glycine. The Thr aldolase activity can be attributed in bacteria to two different enzymes: serine hydroxymethyltransferase (SHMT) and to a low-specificity Thr aldolase (LTA). SHMT-encoding genes are present in all LAB genomes, while genes encoding LTA have yet to be identified. Biochemical and genetic studies have shown that Thr aldolase in LAB is possibly due solely to SHMT enzyme (Chaves et al. 2002). Thr can also be deaminated to 2-oxobutanoate, the precursor compound for the biosynthesis of BCAAs. Deaminase-encoding genes have been found in *L. lactis*, streptococci, *Lact. casei*, *Lact. sakei*, *Leuc. mesenteroides*, and *Ped. pentosaceus*.

### 1.3.7. Biogenic Amines Pathways from Amino Acids

Catabolism of some amino acids can produce biogenic amines (BAs), which can cause food poisoning. BAs are mainly formed by decarboxylation of the amino acids tyrosine, histidine, lysine, and ornithine (Fig. 1.3), from which tyramine, histamine, cadaverine, and putrescine, respectively, are produced. Tyrosine, histidine, and ornithine decarboxylase activities have been reported in LAB species and strains of *Lactobacillus*, *Enterococcus*, *Oenococcus*, *Tetragenococcus*, and *Carnobacterium*. Genes encoding decarboxylase enzymes are found in clusters, which include genes encoding amino acid-BA antiporters, and in some cases (histamine and tyramine) aminoacyl tRNA synthetases. The similar organization of different clusters, their distribution, and their high similarity of sequence suggest a horizontal transference from a common source (review by Fernández and Zúñiga 2006). Lysine decarboxylase has never been identified in LAB.

Arg decarboxylase (ADC) converts Arg into agmatine. This activity has been reported only in *Lactobacillus hilgardii* X1B (Arena and Manca de Nadra 2001). Furthermore, several LAB can use agmatine via the agmatine deiminase pathway to produce putrescine. This pathway is constituted by three enzymes: agmatine deiminase, putrescine carbamoyltransferase, and carbamate kinase. The agmatine deiminase pathway was first described in *Enterococcus faecalis* (Simon and Stalon 1982) and genome analyses and recent results obtained by Lucas et al. (2007) revealed the presence of agmatine deiminase genes in different *Lact. brevis*, *Lact. sakei*, and *Ped. pentosaceus* strains.

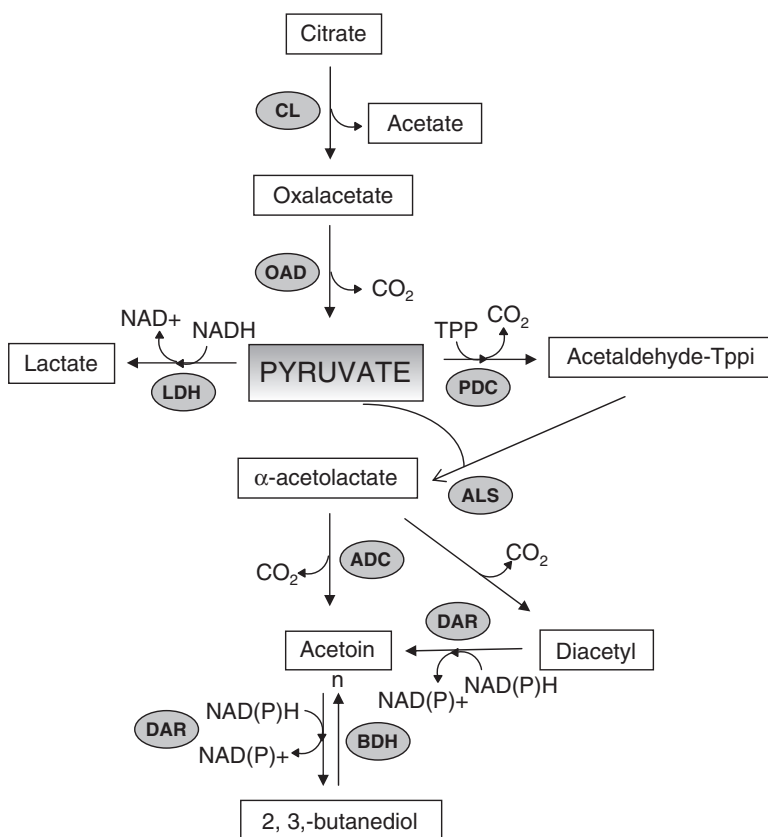
### 1.3.8. Arginine Metabolism

Arginine pathways have been deeply studied in LAB species, although it is not involved in either flavor or BA formation. The most common pathway for the catabolism of arginine in LAB is via the arginine deiminase (ADI) pathway. The ADI

pathway comprises three reactions catalyzed by ADI, ornithine carbamoyl-transferase and carbamate kinase (Fernández and Zúñiga 2006). Degradation of Arg through the ADI pathway results in production of ATP and ammonia. Therefore, the ADI pathway is thought to provide energy and protection against an acidic external pH. Nevertheless, its relevance as an energy source or as a protective system against acidic environments varies among LAB. In general, arginine induces the expression of ADI enzymes and some carbohydrates repress their synthesis, being controlled by catabolite repression.

#### 1.4. Citrate Metabolism and Formation of Aroma Compounds

In addition to sugars, several LAB species have the capability of metabolizing citrate, a process requiring citrate transport, conversion of citrate into oxaloacetate, and then into pyruvate and CO<sub>2</sub> (Fig. 1.4). Citrate fermentation by LAB leads to the production of 4-carbon compounds, such as diacetyl, acetoin and butanediol, which have aromatic properties and impart the typical aroma to many dairy products. Strains of *L. lactis* subsp. *lactis* biovar. *diacetylactis* (*L. diacetylactis*) and species of *Leuconostoc* and



**Figure 1.4.** Citrate metabolism in *Lactococcus* and *Leuconostoc* species. Key for the enzymes: CL, citrate lyase; OAD, oxaloacetate decarboxylase; LDH, lactate dehydrogenase; PDC, pyruvate decarboxylase; ALS, α-acetolactate synthase; ADC, α-acetolactate decarboxylase; DAR, diacetyl acetoin reductase; BDH, 2,3-butanediol dehydrogenase; Tppi, thiamine pyrophosphate.

*Weissella* are used by the fermentation industry as diacetyl producers. Other LAB species such as *Lact. plantarum* and *O. oeni* use the citrate present in raw materials to produce a secondary fermentation in wine, beer, and sausages, which confers off-flavors to the fermented products. Thus, either beneficial or detrimental, the use of citrate by LAB has a great industrial interest.

#### 1.4.1. Citrate Transport

Transport of citrate is performed by specific membrane-associated permeases, and constitutes the limiting step for citrate utilization. Biochemical characterization of the permeases has shown that citrate can be incorporated by diverse mechanisms. Most LAB species internalize citrate by a 2-hydroxycarboxylate type of transporters, which transports dicarboxylic and tricarboxylic acids (Sobczak and Lolkema 2005). Among the citrate transporters of this family, the well-characterized CitS and the CitP from *Lactococcus*, *Leuconostoc*, and *Weissella* are included. CitS is a symporter, which uses a  $\text{Na}^{2+}$ -gradient to transport citrate, whereas CitP is responsible for the antiport of H-citrate<sup>2-</sup> and lactate<sup>1-</sup> generating a membrane potential. In LAB the genes encoding CitP are identified in plasmids and show a 99% nucleotide sequence identity, suggesting recent acquisition by horizontal transfer (Dridet et al. 2004). Genes from different species are located in unrelated plasmids and present a different genetic organization (Sesma et al. 1990; Martín et al. 2000). In contrast, in *Ent. faecalis* and *Streptococcus mutans* citrate is transported by CitM and CitH transporters, respectively, belonging to the recently described family of citrate-metal symporters (CitMHS) (Korithoski et al. 2005; Blancato et al. 2006). Uptake of citrate by these transporters occurs in the form of complexes with cations (either  $\text{Ca}^{2+}$ ,  $\text{Mn}^{2+}$ , or  $\text{Fe}^{3+}$ ). Additionally, the genes encoding CitM and CitH are located in the chromosome, associated to citrate fermentation clusters. Citrate transporters of other types may be found in *Lact. plantarum* (Kleerebezem et al. 2003) and *O. oeni* (Augagneur et al. 2007).

#### 1.4.2. Conversion of Citrate into Pyruvate

Once inside, citrate is converted into acetate and oxaloacetate in a reaction catalyzed by the citrate lyase (CL) enzyme. CL is an enzymatic complex, which catalyzes the conversion of citrate in a multi-reaction process. CL has also been purified from *Leuc. mesenteroides* 195 strain (Bekal et al. 1998), and the CL-coding genes have been cloned from this strain (Bekal et al. 1998), from a plasmid belonging to a *Weissella paramesenteroides* strain (Martín et al. 1999, 2000), and from the chromosome of *L. diacetylactis* CRL264 (Martín et al. 2004).

The second step of citrate metabolism, catalyzed by oxaloacetate decarboxylase (OAD), is the decarboxylation of oxaloacetate, generating pyruvate and  $\text{CO}_2$ . Analysis of the genomes of various LAB indicate that a homolog of the *Klebsiella pneumoniae* OAD, the best characterized bacterial enzyme of this type, is only present in *Strep. mutans*, *Ent. faecalis*, *Lact. sakei*, and *Lact. casei*. In most citrate fermenters, OAD seems to be a 40-kDa polypeptide sharing high amino acid identity with proteins of the malic enzyme family. OAD is encoded by either *citM* in *Weissella* (Martín et al. 2000), or *mae* in all other LAB (Bekal et al. 1998; Bolotin et al. 2001). In agreement, co-transcription of these genes with that encoding CL has been detected in *Leuconostoc*, *Weissella*, and *L. diacetylactis* (Bekal et al. 1998; Martín et al. 2000, 2004).

#### 1.4.3. Conversion of Pyruvate into Aroma Compounds

Metabolism of pyruvate can yield in LAB different end-products such as lactate, formate, acetate, ethanol, and the aroma compounds of four carbons ( $\text{C}_4$  compounds) diacetyl, acetoin, and butanediol (Neves et al. 2005; Fig. 1.4). The metabolic biosynthetic pathway from citrate to diacetyl was revealed in *L. diacetylactis* by use of nuclear magnetic resonance techniques. Ramos et al. (1994) demonstrated that the main route of diacetyl synthesis is via the intermediary  $\alpha$ -acetolactate (Fig. 1.4).  $\alpha$ -Acetolactate synthase (ALS) is the key enzyme in the synthesis of  $\text{C}_4$  compounds by catalyzing the

condensation of two pyruvate molecules to generate  $\alpha$ -acetolactate. Once synthesized,  $\alpha$ -acetolactate is unstable and is readily decarboxylated to acetoin by  $\alpha$ -acetolactate decarboxylase (ALD), or by nonenzymatic decarboxylation (in the presence of oxygen) to diacetyl. Acetoin can also be synthesized from diacetyl by diacetyl reductase (DAR). This enzyme also possesses acetoin reductase activity, yielding 2,3 butanediol from acetoin, while the reverse reaction is catalyzed by 2,3 butanediol dehydrogenase (BDH). The balance between the end-products of citrate fermentation will depend on the redox state of the cells (Bassit et al. 1993).

The genes encoding both ALS, *als* and ALD, *aldB* have been cloned and characterized from *L. diacetylactis* (Marugg et al. 1994; Goupil-Feuillerat et al. 1997). AldB plays a dual role, catalyzing the second step of the acetoin biosynthetic pathway, and regulating the pool of  $\alpha$ -acetolactate in the cell during BCAA metabolism. Transcriptional analysis of the *aldB* expression revealed that this gene forms an operon with *leu*, *ilv*, and *aldR* genes. In fact, the *aldB* is located downstream of genes encoding enzymes involved in the biosynthesis of BCAAs (leucine, valine, and isoleucine) and precedes the *aldR* gene, which encodes a putative regulator whose function has not been completely elucidated (Goupil-Feuillerat et al. 1997). This second role is very important as BCAAs are essential for protein synthesis and, consequently, the expression of this enzyme is strictly regulated at both the transcriptional and post-transcriptional levels (Goupil-Feuillerat et al. 1997, 2000).

DAR catalyzes two stages of citrate metabolism: the conversion of diacetyl into acetoin and the reduction of acetoin to 2, 3-butanediol. Conversion of 2,3-butanediol into acetoin is catalyzed by BDH. In *L. diacetylactis*, two proteins seem to possess DAR and BDH activities. The DAR enzyme of *Leuconostoc pseudomesenteroides* is encoded by the plasmidic *butA* gene (Rattray et al. 2000). Bioinformatic analysis of the *butA* gene from this species allowed identifying the *butA* gene in the *L. diacetylactis* IL1403 genome (García-Quintáns et al. 2008). Both enzymes share 81% of amino acid identity. Preceding the *L. diacetylactis* IL1403 *butA*

is the *butB* gene, whose product was annotated as BDH in the lactococcal genome sequence. BDH belongs to the Zn-containing alcohol dehydrogenases. The genes *butB* and *butA* are also linked to the *L. lactis* subsp. *cremoris* (MG1363 and SK11) chromosome. However, in other LAB species, such as *Leuc. pseudomesenteroides*, *butA* is carried on plasmids (Rattray et al. 2003), which may facilitate its horizontal transfer. The two DARs purified from lactococci have less affinity for 2,3-butanediol than for diacetyl and acetoin. They both have optimal alkaline and acidic pH, respectively, for its BDH and DAR activities. Therefore, it is conceivable that at acidic pH an increase of 2,3-butanediol occurs; a fact that has experimentally been observed in *L. diacetylactis* (Ramos et al. 1994).

#### 1.4.4. Regulation of Citrate Utilization

The biochemical machinery for sensing, transport, and utilization of citrate is grouped into operons, showing highly conserved components in all LAB species. A *citMCDEFXG* (*cit*) operon has been also identified in the *L. diacetylactis* chromosome. However, the citrate permease *citP* is not part of the operon; instead, it is encoded on the citrate transport operon, *citQRP*, located in the citrate plasmid (López de Felipe et al. 1995). Similarly, a *cit* operon (*citMCDEFGRP*) encoding CitM, CL, and CitP is found in *W. paramesenteroides*. Upstream of this operon is the divergently transcribed *citI* gene (Martín et al. 1999, 2000). Two unique and different models of regulation for citrate metabolism have been described in LAB. In *L. diacetylactis*, specific transcriptional activation of the promoters controlling *cit* operons takes place at low pH; this activation is thought to provide an adaptive response to acidic stress. In *W. paramesenteroides*, the CitI transcriptional regulator functions as a citrate-activated switch allowing the cell to optimize generation of metabolic energy.

#### 1.4.5. Bioenergetics of Citrate Metabolism

During growth in milk, *L. diacetylactis* metabolizes lactose-producing lactic acid, which is exchanged

for citrate during excretion by the antiporter CitP. As a homofermentative organism, *L. diacetylactis* converts glucose into lactate, producing 2 moles of ATP per mol of glucose metabolized. The NAD<sup>+</sup> consumed in the first steps of the pathway is regenerated during the transformation of pyruvate into lactate, thus maintaining the redox potential. In the presence of glucose and citrate, each mol of citrate produces one mol of pyruvate without generating NADH. This excess of pyruvate is diverted to the synthesis of  $\alpha$ -acetolactate and, subsequently, to the production of aroma compounds. The utilization of citrate results in a growth advantage for *L. diacetylactis* when the medium has a low pH (García-Quintáns et al. 1998; Magni et al. 1999; Martín et al. 2004). Recent results have shown that contrary to what has been argued, the beneficial effect of citrate on growth under acid stress is not primarily due to a concomitant alkalization of the medium (detoxification effect), but seems to stem from less expenditure of the ATP obtained from glucose to achieve pH homeostasis (Sánchez et al. 2008).

Glucose metabolism in heterofermentative *Leuconostoc*, *Oenococcus*, and *Lactobacillus* species takes place through the pentose phosphate pathway, a heterolactic fermentation characterized by lactate, ethanol, and acetate as end-products (Zaunmüller et al. 2006). In this case the main part of the acetylphosphate formed during the glucose degradation is reduced to ethanol by the phosphoketolase pathway, regenerating the NAD<sup>+</sup> consumed. However, the presence of citrate together with glucose provokes a shift to the heterofermentative route. When citrate is present, the NAD<sup>+</sup> is regenerated during the pyruvate reduction to lactate. The acetylphosphate is therefore converted to acetate by means of the acetate kinase, generating an extra mol of ATP (Sobczak and Lolkema 2005; Fig. 1.1C).

### 1.5. Functional Genomics and Metabolism

The recently completed genome sequence of LAB species (Mayo et al. 2008) creates an excellent opportunity to examine how metabolic pathways

function in a global perspective. As microbial genomes reflect the metabolism, physiology, biosynthetic capabilities, and adaptability of the organisms to varying conditions and environments, the availability of genome sequences has also expanded our knowledge of LAB's metabolic potential and bioprocessing capabilities (Teusink and Smid 2006). As an example in the following paragraph, the respiration capacity of *L. lactis* and its industrial-derived applications are briefly presented (Duwat et al. 2001; Gaudu et al. 2002).

Sugar fermentation was long considered to represent the sole means of energy metabolism available in LAB, producing organic acids (mainly lactic acid) as a final product. Early evidence by Sijpesteijn in 1970 for the respiratory capability of LAB was largely overlooked (as reported in Duwat et al. 2001), and only after analysis of the complete genome sequence of *L. lactis* IL1403 (Bolotin et al. 2001) a respiratory capacity for this species in the presence of exogenous heme was first envisioned (Duwat et al. 2001). Subsequent research confirmed that *L. lactis* certainly has the ability to respire in the presence of oxygen (Duwat et al. 2001; Gaudu et al. 2002), provided the growth medium contains heme because this bacterium does not have a functional biosynthetic pathway for this compound (Bolotin et al. 2001). Under respiratory conditions, acetate, acetoin, and diacetyl are produced from pyruvate at the expense of lactic acid (Duwat et al. 2001). An important consequence of respiration is a more efficient conversion of the carbon source into biomass, resulting in higher cell yields and increased survival after growth. The initial results obtained with the well-characterized laboratory strain *L. lactis* IL1403 were optimized for different industrial *L. lactis* strains and also for *Leuconostoc* species (Pedersen et al. 2005). These results are of industrial significance and allowed the development of a patented process for the production of LAB starter cultures (Duwat et al. 1998). *L. lactis* strains were also assayed in aeration in the absence of added heme to distinguish simply aeration from true respiration (Pedersen et al. 2005). Numerous genes were differentially expressed under these two conditions. Approximately half of these genes have unknown



function, indicating that more research is still needed to fully understand the physiology of respiration in this species. However, increased biomass after aerated incubation in the presence of heme was not obtained for *Strep. thermophilus*, *Lact. bulgaricus*, and *Lact. helveticus*. Analysis of the complete genome sequences of all these species (Bolotin et al. 2004; van de Guchte et al. 2006; Callanan et al. 2008) does not reveal the presence of genes for cytochrome oxidase or for the biosynthesis of quinones, features which are believed to be essential for respiration (Gaudu et al. 2002).

Starter cultures obtained by the respiration technology have been assayed in pilot scale tests for Cheddar cheese production and compared with starters produced under standard conditions (Pedersen et al. 2005). Manufacture parameters were all within the normal range. Indeed, sensory differences were not perceived by trained panels after maturation. Industrial scale trials of Cheddar, Feta, and Cottage cheese have already been done, and again no significant differences were seen in the manufacturing parameters, cheese microbiology, chemistry, texture, or flavor development (Pedersen et al. 2005).

## 1.6. Metabolic Engineering of LAB Metabolism

Cryptic plasmids from many LAB species provided the platform for the development of sophisticated genetic tools (e.g., vectors for cloning [including food-grade vectors], expression, and integration; Mills et al. 2006). Of note is the nisin-controlled gene expression system, which has been in use for more than 10 years (Mierau and Kleerebezem 2005). These and other genetic tools allowed a first engineering and rerouting of LAB metabolism (de Vos and Hugenholtz 2004; Hugenholtz 2008). Among the many examples that can be mentioned with regard to illustrating the high potential of engineering the metabolism of LAB species, the conversion of *L. lactis* into a homoalanine producer as the only fermentation end-product was reported as early as in 1999 (Hols et al. 1999). The conversion of this

species into a high diacetyl producer by combining NADH-oxidase overproduction and  $\alpha$ -ALD inactivation has also been achieved (Hugenholtz et al. 2000). The other important aroma compound in dairy products is acetaldehyde. It is produced by both *Lact. delbrueckii* and *Strep. thermophilus* in yoghurt fermentation; however, it is hardly produced as a fermentation end-product in *L. lactis*. In lactococcal cells, acetaldehyde can be converted from acetyl coenzyme A by the action of aldehyde dehydrogenase (ADH; Fig. 1.1B). Recently, the efficient rerouting of pyruvate toward acetaldehyde in *L. lactis* has been achieved by functional expression of a pyruvate decarboxylase gene from *Zymomonas mobilis* and the endogenous NOX gene (Bongers et al. 2005).

The controlled overexpression of key genes in the folate biosynthesis cluster has been used to overproduce this vitamin in *L. lactis* cells (Sybesma et al. 2003). Cloning and expression of the whole folate operon in *Lact. gasseri* converted this folate-consumer species into a folate producer (Wegkamp et al. 2004). Furthermore, *L. lactis* has also been used for the production of the nutraceutical compound mannitol (Wisselink et al. 2004). More recently, by reverting the sorbitol catabolic pathway in a *Lact. plantarum*, mutant deficient in both L- and D-lactate dehydrogenase activities has been forced to become a high-level sorbitol producer from fructose-6-phosphate (Ladero et al. 2007).

Overexpression of genes encoding components of the proteolytic system has also been accomplished in several LAB species under cheese conditions (Christensen et al. 1995; Tuler et al. 2002). Cheeses produced with the recombinant overexpressing-strains have frequently received higher scores than those made with the original strains. However, it has been repeatedly reported that amino acid degradation is the actual process controlling flavor formation in cheese from proteins (for recent reviews see Smit et al. 2005; Fernández and Zúñiga 2006). The availability of whole genomes of many LAB species would allow the building of complete metabolic structural models. Data derived from genome sequence information could also be used as tools to direct metabolic engineering strategies to

improve LAB as cell factories (Oliveira et al. 2005; Smid et al. 2005).

## 1.7. Conclusions

Knowledge of the metabolism of industrially important LAB species has been gathered during the last 25 years. In particular, the metabolism of carbohydrates, citrate, and amino acids, and the key components produced during their degradation are all known. Rudimentary genetic tools have already been used to engineer and reroute some metabolic pathways involved in the synthesis of the desirable compounds that define and are sought for in different fermented products. At present the new techniques and disciplines emerging in the post-genomic era, such as genomics, proteomics, metabolomics, and system biology, open new avenues for interpretation of biological data, making possible the development of new predictive models. In combination with classical and molecular techniques, these new methods will be invaluable in the rational optimization of LAB metabolism in order to obtain more tasteful, aromatic, and safer traditional and new fermented products. This knowledge will ultimately allow a more rational use of LAB species not only in traditional fermentations but also in novel bioprocessing, probiotic, and biotechnological applications.

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## Chapter 2

# Genomics of Lactic Acid Bacteria: The Post-genomics Challenge—From Sequence to Function

M. Andrea Azcarate-Peril and Todd R. Klaenhammer

*Following the publication of the complete genome sequence of *Lactococcus lactis* strain IL1403, intensive efforts began to determine the entire genome sequences of many members of the lactic acid bacteria (LAB). This functionally and phylogenetically related group of bacteria is widely used in a variety of applications that include food fermentation, production of industrial chemicals and metabolites, use as probiotics, and recently as promising candidates for delivery of biotherapeutics and vaccines. This chapter overviews the main aspects of the genomics revolution that took place during the past decade in LAB. The impact of genome sequencing on the taxonomy of this group will be briefly reviewed. Furthermore, important genomic features on gene acquisition, loss, and degradation will be discussed in terms of the evolution and ecology of LAB. This review will also provide an overview of recent functional genomics studies that have identified new functions and linked the responsible genes and operons to important phenotypic properties.*

### 2.1. The Genomics Revolution

Only 6 years had elapsed since the completion of the first bacterial sequenced genome, *Haemophilus influenzae* Rd (Fleischmann et al. 1995) to the public release of the first lactic acid bacteria (LAB) genome sequenced in 2001. In fact, by 2001 four genomes belonging to LAB were released, three of them pathogens (*Streptococcus pyogenes* M1 GAS [Ferretti et al. 2001], *Streptococcus pneumoniae*

TIGR4 [Tettelin et al. 2001], and *S. pneumoniae* R6 [Hoskins et al. 2001]). The fourth one was a food-grade organism, *Lactococcus lactis* subsp. *lactis* IL1403 (Bolotin et al. 2001).

By September 2008 there were 128 LAB genomes (complete and in progress) publicly available at the National Center for Biotechnology Information (NCBI; <http://www.ncbi.nlm.nih.gov/>; Table 2.1). This chapter will focus on the genomics of nonpathogenic LAB. Furthermore, the genus *Bifidobacterium*, weakly phylogenetically related and traditionally included in the LAB group (Felis and Dellaglio 2007), will not be included in this analysis. The genera currently included in the LAB group are *Lactobacillus*, *Weissella*, *Carnobacterium*, *Streptococcus*, *Enterococcus*, *Lactococcus*, *Vagococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, and *Tetragenococcus* (Holzapfel et al. 2001).

The availability of the genome sequences of multiple species, and even multiple strains within species, has revealed new gene functions and applications of the LAB. Additionally, this new explosion of information has brought into discussion the environments from which each bacterium evolved, in order to explain newly characterized functionalities. A systems-biology approach (Fig. 2.1) with data input from the most important aspects of the system, which are the list of parts, the connection between those parts, and the localization of the sum of the parts with its connections in space and time (Raes and Bork 2008), will pave the way to

**Table 2.1.** Lactic acid bacteria (LAB) genomes and genome projects. Modified from [http://www.ncbi.nlm.nih.gov/genomes/MICROBES/microbial\\_taxtree.html](http://www.ncbi.nlm.nih.gov/genomes/MICROBES/microbial_taxtree.html). Nonpathogenic LAB are in bold.

Organism	* Size	GC	Habitat	Pathogenic in	Disease
<i>Streptococcus gordonii</i> str. Challis substr. CH1	2.2	40.5	Host-associated	Human	Caries and periodontal diseases
<i>Streptococcus equi</i>	* 2.22	41	Host-associated	Equine	Strangles
<i>Enterococcus faecalis</i> V583	3.36	37.4	Multiple	Human	Bacteremia, endocarditis, urinary tract infection
<i>Enterococcus faecium</i> DO	* 2.85	37.9	Multiple	Human	Urinary tract infection, bacteremia, endocarditis
<b><i>Lactococcus lactis</i> subsp. <i>lactis</i> II1403</b>	<b>2.4</b>	<b>35.3</b>	<b>Multiple</b>	<b>No</b>	
<b><i>Lactobacillus acidophilus</i> NCFM</b>	<b>2</b>	<b>34.7</b>	<b>Multiple</b>	<b>No</b>	
<b><i>Lactobacillus gasseri</i> ATCC 33323</b>	<b>1.9</b>	<b>35.3</b>	<b>Host-associated</b>	<b>No</b>	
<i>Streptococcus mitis</i> NCTC 12261	* 1.84	40	Host-associated	Human	Bacterial endocarditis
<i>Streptococcus pyogenes</i> M1 GAS	1.9	38.5	Host-associated	Human	Wide range of infections
<i>Streptococcus pyogenes</i> str. Manfredo	1.8	38.6	Host-associated	Human	Wide range of infections
<i>Streptococcus pneumoniae</i> TIGR4	2.2	39.7	Multiple	Human	Pneumonia, Meningitis, Bacteremia, Sinusitis, Otitis media, Conjunctivitis
<i>Streptococcus pneumoniae</i> R6	2.04	39.7	Multiple	Human	Pneumonia
<i>Streptococcus pyogenes</i> MGAS8232	1.9	38.5	Host-associated	Human	Wide range of infections
<i>Streptococcus pneumoniae</i> 670-6B	* 2.16	40	Multiple	Human	Pneumonia
<i>Streptococcus pyogenes</i> SSI-1	1.9	38.6	Host-associated	Human	Wide range of infections
<i>Streptococcus pyogenes</i> MGAS315	1.9	38.6	Host-associated	Human	Wide range of infections
<b><i>Leuconostoc mesenteroides</i> subsp. <i>mesenteroides</i> ATCC 8293</b>	<b>2.04</b>	<b>37.7</b>	<b>Multiple</b>	<b>No</b>	
<b><i>Oenococcus oeni</i> PSU-1</b>	<b>1.8</b>	<b>37.9</b>	<b>Multiple</b>	<b>No</b>	
<i>Streptococcus agalactiae</i> A909	2.13	35.6	Host-associated	Human	Septicemia, pneumonia, and meningitis
<i>Streptococcus agalactiae</i> 2603V/R	2.2	35.6	Host-associated	Human	Septicemia, pneumonia, and meningitis
<i>Streptococcus mutans</i> UA159	2.03	36.8	Host-associated	Human	Dental caries
<i>Streptococcus agalactiae</i> NEM316	2.2	35.6	Host-associated	Human	Neonatal GBS meningitis
<i>Streptococcus pneumoniae</i> 23F	* 2.13	40	Multiple	Human	Pneumonia
<i>Streptococcus suis</i> P1/7	* 1.83	40	Multiple	Human, Swine	Meningitis, endocarditis, septicemia, and arthritis
<i>Streptococcus uberis</i> 0140J	* 1.82	40	Multiple	Cattle	Mastitis
<b><i>Lactobacillus plantarum</i> WCFS1</b>	<b>3.34</b>	<b>44.4</b>	<b>Host-associated</b>	<b>No</b>	
<b><i>Pediococcus pentosaceus</i> ATCC 25745</b>	<b>1.8</b>	<b>37.4</b>	<b>Multiple</b>	<b>No</b>	<b>None</b>
<b><i>Lactococcus lactis</i> subsp. <i>cremoris</i> SK11</b>	<b>2.56</b>	<b>35.8</b>	<b>Multiple</b>	<b>No</b>	
<b><i>Lactobacillus casei</i> ATCC 334</b>	<b>2.93</b>	<b>46.6</b>	<b>Multiple</b>	<b>No</b>	
<b><i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i> ATCC BAA-365</b>	<b>1.9</b>	<b>49.7</b>	<b>Multiple</b>	<b>No</b>	
<b><i>Lactobacillus brevis</i> ATCC 367</b>	<b>2.35</b>	<b>46.1</b>	<b>Multiple</b>	<b>No</b>	
<i>Streptococcus sobrinus</i> 6715	* 3.53		Host-associated	Human	Dental caries
<b><i>Lactobacillus johnsonii</i> NCC 533</b>	<b>2</b>	<b>34.6</b>	<b>Host-associated</b>	<b>No</b>	
<i>Streptococcus suis</i> 89/1591	* 1.98	41	Multiple	Human, Swine	Meningitis, endocarditis, septicemia, and arthritis
<i>Streptococcus pyogenes</i> MGAS10394	1.9	38.7	Host-associated	Human	Wide range of infections
<b><i>Streptococcus thermophilus</i> LMG 18311</b>	<b>1.8</b>	<b>39.1</b>	<b>Multiple</b>	<b>No</b>	
<b><i>Streptococcus thermophilus</i> CNRZ1066</b>	<b>1.8</b>	<b>39.1</b>	<b>Multiple</b>	<b>No</b>	
<b><i>Lactobacillus salivarius</i> UCC118</b>	<b>2.1</b>	<b>33</b>	<b>Host-associated</b>		

Table 2.1. Continued

Organism	* Size	GC	Habitat	Pathogenic in	Disease
<i>Streptococcus pyogenes</i> M49 591	* 1.33	38.3	Host-associated	Human	Wide range of infections
<i>Lactobacillus helveticus</i>		<b>37.1</b>	<b>Multiple</b>	<b>No</b>	
<i>Lactobacillus reuteri</i> 100-23		<b>38.7</b>	<b>Host-associated</b>	<b>No</b>	
<i>Lactobacillus sakei</i> subsp. <i>sakei</i> 23K	<b>1.9</b>	<b>41.3</b>	<b>Multiple</b>		
<i>Streptococcus thermophilus</i> LMD-9	<b>1.91</b>	<b>39.1</b>	<b>Multiple</b>	<b>No</b>	<b>None</b>
<i>Streptococcus pyogenes</i> MGAS6180	1.9	38.4	Host-associated	Human	Wide range of infections
<i>Streptococcus pyogenes</i> MGAS5005	1.84	38.5	Host-associated	Human	Wide range of infections
<i>Streptococcus pneumoniae</i> TIGR4		39.6	Multiple	Human	Pneumonia, Meningitis, Bacteremia, Sinusitis, Otitis media, Conjunctivitis
<i>Streptococcus sanguinis</i> SK36	2.4	43.4	Host-associated	Human	Endocarditis
<i>Streptococcus agalactiae</i> 18RS21		36.9	Host-associated	Human	Septicemia, pneumonia, and meningitis
<i>Streptococcus agalactiae</i> 515		35.3	Host-associated	Human	Septicemia, pneumonia, and meningitis
<i>Streptococcus agalactiae</i> CJB111		35.6	Host-associated	Human	Septicemia, pneumonia, and meningitis
<i>Streptococcus agalactiae</i> H36B		35.6	Host-associated	Human	Septicemia, pneumonia, and meningitis
<i>Streptococcus agalactiae</i> COH1		35.5	Host-associated	Human	Septicemia, pneumonia, and meningitis
<i>Streptococcus salivarius</i> JIM8777		37-40	Host-associated	Human	Opportunistic, blood infection
<i>Streptococcus salivarius</i> JIM8780			Host-associated	Human	Opportunistic infections
<i>Leuconostoc gasicomitatum</i>			<b>Specialized</b>		
<i>Lactobacillus reuteri</i> F275	<b>2</b>	<b>38.9</b>		<b>No</b>	
<i>Leuconostoc citreum</i> KM20	<b>1.9</b>	<b>38.9</b>		<b>No</b>	<b>None</b>
<i>Streptococcus pyogenes</i> MGAS9429	1.84	38.5	Host-associated	Human	Wide range of infections
<i>Streptococcus pyogenes</i> MGAS10270	1.9	38.4	Host-associated	Human	Wide range of infections
<i>Streptococcus pyogenes</i> MGAS2096	1.86	38.7	Host-associated	Human	Wide range of infections
<i>Streptococcus pyogenes</i> MGAS10750	1.94	38.3	Host-associated	Human	Wide range of infections
<i>Streptococcus pneumoniae</i> D39	2	39.7	Multiple	Human	Pneumonia
<i>Streptococcus iniae</i> 9117	* 1.8		Host-associated	Fish, Human	Meningitis, Cellulitis
<i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i> ATCC 11842	<b>1.86</b>	<b>49.7</b>	<b>Multiple</b>	<b>No</b>	
<i>Streptococcus suis</i> 05ZYH33	2.1	41.1	Multiple	Swine, Human	Meningitis, endocarditis, septicemia, and arthritis
<i>Streptococcus suis</i> 98HAH33	2.1	41.1	Specialized	Swine, Human	Meningitis, endocarditis, septicemia, and arthritis
<i>Streptococcus suis</i> 05HAH33			Multiple	Swine, Human	Meningitis, endocarditis, septicemia, and arthritis
<i>Lactobacillus helveticus</i> DPC 4571	<b>2.1</b>	<b>37.1</b>	<b>Multiple</b>	<b>No</b>	<b>None</b>
<i>Streptococcus pneumoniae</i> SP3-BS71		39.8	Multiple	Human	Pneumonia
<i>Streptococcus pneumoniae</i> SP6-BS73		39.6	Multiple	Human	Pneumonia
<i>Streptococcus pneumoniae</i> SP9-BS68		39.6	Multiple	Human	Pneumonia
<i>Streptococcus pneumoniae</i> SP11-BS70		39.7	Multiple	Human	Pneumonia
<i>Streptococcus pneumoniae</i> SP14-BS69		39.6	Multiple	Human	Pneumonia
<i>Streptococcus pneumoniae</i> SP18-BS74		39.7	Multiple	Human	Pneumonia
<i>Streptococcus pneumoniae</i> SP19-BS75		39.6	Multiple	Human	Pneumonia
<i>Streptococcus pneumoniae</i> SP23-BS72		39.7	Multiple	Human	Pneumonia
<i>Lactococcus garvieae</i> ATCC 49156			Host-associated	Fish, Shellfish, Cattle, Human	Lactococcosis
<i>Lactococcus garvieae</i> Lg2			Host-associated	Fish, Shellfish, Cattle, Human	Lactococcosis

Table 2.1. Continued

Organism	* Size	GC	Habitat	Pathogenic in	Disease
<i>Streptococcus suis</i> GZ1			Host-associated	Swine, Human	Meningitis, arthritis, pneumonia
<i>Lactococcus lactis</i> subsp. <i>cremoris</i> MG1363	2.5	35.7	Multiple	No	None
<i>Lactobacillus fermentum</i> IFO 3956	2.1	51.5	Multiple		
<i>Lactobacillus reuteri</i> F275	2	38.9	Host-associated	No	None
<i>Carnobacterium</i> sp. AT7		35.3	Specialized		
<i>Streptococcus infantarius</i> subsp. <i>infantarius</i> ATCC BAA-102		37.6	Host-associated	No	
<i>Streptococcus pneumoniae</i> SP195		39.7	Multiple	Human	Pneumonia
<i>Streptococcus pneumoniae</i> CDC3059-06		39.9	Multiple	Human	Pneumonia, Meningitis, Bacteremia, Sinusitis, Otitis media, Conjunctivitis
<i>Streptococcus pneumoniae</i> CDC0288-04		39.9	Multiple	Human	Pneumonia, Meningitis, Bacteremia, Sinusitis, Otitis media, Conjunctivitis
<i>Streptococcus pyogenes</i> NZ131		38	Host-associated	Human	Wide range of infections
<i>Streptococcus pneumoniae</i> CDC1873-00		39.6	Multiple	Human	Pneumonia, Meningitis, Bacteremia, Sinusitis, Otitis media, Conjunctivitis
<i>Streptococcus pneumoniae</i> CDC1087-00		39.8	Multiple	Human	Pneumonia, Meningitis, Bacteremia, Sinusitis, Otitis media, Conjunctivitis
<i>Enterococcus faecalis</i> OG1RF		37.8	Multiple	Human	Urinary infection, Bacteremia, Endocarditis
<i>Streptococcus pneumoniae</i> MLV-016		39.9	Multiple	Human	Pneumonia
<i>Streptococcus canis</i> FSL Z3-227			Host-associated	Animal, Human	Mastitis, toxic shock, and necrotizing fasciitis
<i>Streptococcus pneumoniae</i> Hungary19A-6	2.2	39.6	Multiple	Human	Pneumonia
<i>Streptococcus pneumoniae</i> Taiwan19F-14			Multiple	Human	Pneumonia
<i>Streptococcus pneumoniae</i> JJA			Multiple	Human	Pneumonia
<i>Streptococcus pneumoniae</i> 70585			Multiple	Human	Pneumonia
<i>Streptococcus pneumoniae</i> SP14-BS292			Multiple	Human	Pneumonia
<i>Streptococcus pneumoniae</i> SP9v-BS293			Multiple	Human	Pneumonia
<i>Streptococcus pneumoniae</i> 459-2			Multiple	Human	Pneumonia
<i>Streptococcus pneumoniae</i> 459-5			Multiple	Human	Pneumonia
<i>Lactobacillus casei</i> str. Zhang		46.6	Multiple	No	
<i>Streptococcus mutans</i> NN2025			Host-associated	Human	Dental caries, endocarditis
<i>Lactobacillus buchneri</i> NRRL B-30929			Multiple	No	
<i>Streptococcus pneumoniae</i> G54	2.1	39.6	Host-associated	Human	Pneumonia
<i>Streptococcus pneumoniae</i> BS457			Multiple	Human	Pneumonia
<i>Streptococcus pneumoniae</i> BS458			Multiple	Human	Pneumonia
<i>Streptococcus pneumoniae</i> BS397			Multiple	Human	Pneumonia
<i>Streptococcus pneumoniae</i> CGSP14	2.2	39.5	Multiple	Human	Pneumonia
<i>Lactobacillus rhamnosus</i> HN001	0.04	46.7	Multiple	No	
<i>Enterococcus faecium</i> E980		37.9	Multiple	Human	Opportunistic infections
<i>Enterococcus faecium</i> E1071		37.9	Multiple	Human	Opportunistic infections
<i>Enterococcus faecium</i> E1162		37.9	Multiple	Human	Opportunistic infections
<i>Lactobacillus paracasei</i> subsp. <i>paracasei</i> 8700:2		46.2	Multiple	No	



Table 2.1. Continued

Organism	* Size	GC	Habitat	Pathogenic in	Disease
<b><i>Lactobacillus casei</i> BL23</b>	<b>3.1</b>	<b>46.3</b>	<b>Specialized</b>	<b>No</b>	
<i>Enterococcus faecium</i> E1636		37.9	Multiple	Human	Opportunistic infections
<i>Enterococcus faecium</i> E1679		37.9	Multiple	Human	Opportunistic infections
<i>Enterococcus faecalis</i> TX0104			Multiple	Human	Urinary infection, Bacteremia, Endocarditis
<i>Enterococcus faecalis</i> HH22			Multiple	Human	Urinary infection, Bacteremia, Endocarditis
<i>Enterococcus faecium</i> DO		37.9	Multiple	Human	Urinary tract infection, bacteremia, endocarditis
<b><i>Lactobacillus crispatus</i> JV V101</b>					
<b><i>Lactobacillus reuteri</i> SD2112</b>					
<i>Streptococcus bovis</i> TX20005					
<i>Streptococcus iniae</i> 9117					
<i>Enterococcus faecium</i> U0317			Multiple	Human	Opportunistic infections
<i>Streptococcus equi</i> subsp. <i>zooepidemicus</i> MGCS10565	2	41.8	Host-associated	Animals, Human	Opportunistic infections
<b><i>Lactobacillus gasseri</i> MV-22</b>					
<b><i>Lactobacillus jensenii</i> 1153</b>					

modeling complete microbial systems, including novel capabilities in context with their adaptation to natural environments. Within the LAB world, data availability for the first dimension of information (the list of parts) is ample, including genome, pathways, and cell information as well as characterization of the local environment. In fact, 1D information has had a big impact on LAB systems biology and will allow in the near future the ability to obtain additional 2D information to understand the relationships and connections between the parts.

Functional genomics studies conducted over the last 10 years, as well as studies currently under way, are key to developing a systems biology approach to map interactions within local ecosystems and between distinctly different environments.

## 2.2. Genomic Records of LAB Adaptation to the Environment

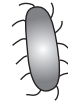
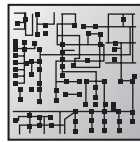
Traditionally, LAB have been associated with dairy products (Cogan et al. 2007), plants (Di Cagno et al. 2008), meats (Hugas et al. 2003; Rantsiou and Cocolin 2006; Tanasupawat et al. 2008), alcoholic beverages (Mills et al. 2005; Moreno-Arribas and Polo 2005), and living organisms, including humans

(Reuter 2001; Azcarate-Peril et al. 2008; Busconi et al. 2008; Laukova et al. 2008; Nazef et al. 2008; Walter 2008).

### 2.2.1. LAB Evolution in Dairy Environment

One of the most prominent examples of the adaptation of a microorganism is illustrated by the evolution of some LAB to the dairy environment. Genomic and metabolic simplification through gene loss or degradation is a recurring theme following the adaptation to milk, a nutritious environment that provides lactose as the main carbohydrate source, casein as the main source of proteins, and most vitamins and minerals. As an example, the most important characteristics of the genome sequence of *Lactobacillus helveticus* DPC4571 (Callanan et al. 2008), a Swiss cheese isolate commonly used as starter and adjunct culture in cheese manufacture, are a predicted dependency on external supplies of amino acids and cofactors similar to that described for closely related gastrointestinal (GI) tract isolates, *Lactobacillus acidophilus* (Altermann et al. 2005) and *Lactobacillus johnsonii* (Pridmore et al. 2004), a high peptidolytic activity, and the ability to lyse rapidly in the cheese matrix, features that play a

A



System	Biomolecule (protein, DNA, sugar, or lipid)	Pathway or process	Cell or microorganism	Ecosystem (local)	Earth
1D information (parts list)	+++	+++	+++	++	X
2D information (interactions)	+++	+++	++	+	X
3D information (spatial)	+++	++	+	+	+
4D information (temporal)	++	++	++	+	+

B

Promising technologies that provide global, high-throughput readouts for ecosystems biology				Ecosystem network analysis
Level of measurement	Molecules	Genes or proteins	Cells or individuals	Ecosystem
1D information (parts list)	Chemical profiling and meta-metabolomics	Metagenomics and single-cell sequencing	Massive parallel 16S tag sequencing	
2D information (interactions)				
3D and 4D information (spatial and temporal)	Chemical profiling and meta-metabolomics	Meta-transcriptomics and meta-proteomics	Phylochips	

**Figure 2.1.** A. Different spatial scales at which systems biology can be performed. The columns show data availability for each scale and the rows indicate the aspect of the system that is targeted by the data. (+++), ample data available and good knowledge of the system aspect; (++) , a number of high-throughput data sets available and fair knowledge of the system aspect, but more data are still needed to build comprehensive models; (+), a few scattered non-high-throughput data sets available and model building is restricted to case studies; (x), almost no data available; B. At the ecosystem scale, readouts are available at different levels: molecules (ranging from trace elements to small signaling compounds to metabolism intermediates), genes or proteins, and cells or individuals. Here, we show some of the more promising high-throughput approaches to the generation of data that would facilitate ecosystems biology. No high-throughput tools are currently available that can map interactions, and this information will need to be inferred from other data sources (Raes and Bork 2008). Reprinted with permission of McMillan Publishers Ltd.

**Table 2.2.** Components of the *Lactobacillus helveticus* CNRZ32 proteolytic enzyme system: A look before and after genome sequence determination (Cogan et al. 2007). Reproduced with permission.

Genes identified and characterized before sequencing project	Genes identified upon completion of the draft genome sequence
<b>Proteinases</b>	
<i>prtH</i>	<i>prtH2</i> plus 9 additional proteases
<b>Endopeptidases</b>	
<i>pepE</i> , <i>pepO</i> , <i>pepO2</i>	<i>pepE2</i> , <i>pepF</i> , <i>pepO3</i> , plus 2
endopeptidases	glycoproteins
<b>Aminopeptidases</b>	
<i>pepC</i> , <i>pepN</i> , <i>pepX</i>	<i>pepC2</i> plus 7 additional
	aminopeptidases
<b>Di-Tripeptidases</b>	
<i>pepD</i> , <i>pepI</i> , <i>pepQ</i> , <i>pepR</i>	<i>pepD2</i> , <i>pepD3</i> , <i>pepD4</i> , <i>pepQ2</i> , <i>pepT1</i> , and <i>pepT2</i>
<b>Other</b>	
	Oligo- and di-tripeptide transport
	systems: <i>oppA</i> , <i>oppA2</i> ,
	<i>oppB-D</i> , <i>oppF</i> , and <i>dtpA</i> ,
	<i>dtpA2</i> , and <i>dtpT</i>
	Multiple amino acid transporters

critical part in cheese ripening. Also an unusually high number of insertion sequence (IS) elements suggests that horizontal gene transfer may have played a very important role in the origin of LAB that are specialized for growth in milk (Bolotin et al. 2004; Makarova et al. 2006). The draft sequence of another strain of *Lact. helveticus*, CNRZ32, characterized more essential components of the proteolytic enzyme system that confirmed and expanded the knowledge of this system in this bacterium (Table 2.2; Cogan et al. 2007).

Transcriptome profiling tools have recently begun to define the relationship between LAB and the milk environment. Microarray technology confirmed the overexpression of several members of the proteolytic system in cultures of *L. helveticus* CNRZ32 growing in milk versus a complex medium, including previously characterized genes (*pepE*, *pepN*, *pepR*, *pepO2*, *pepO*, *pepX*) and genes identified by comparative genomics (*prtH2* and the *opp* operon (Smeianov et al. 2007). In *L. acidophilus* NCFM, transcriptome analysis during growth in milk identified similar members of the proteolytic system as well as a two-component regulatory system involved in the regulation of oligopeptide transport in this probiotic bacteria (Azcarate-Peril et al. 2005, 2009).

## 2.2.2. LAB Evolution in Vegetable and Meat Environments

The microbiota of fresh vegetables normally consists of Gram (–) aerobic bacteria, yeasts, and a lower number of LAB (Harris 1998). However, varying conditions of anaerobiosis, moisture levels, concentration of salt, and temperature result in changes in the population balance and select for spontaneous fermentation by LAB. The same process occurs in meat products where LAB are initially present at low numbers ( $10^2$ – $10^3$  colony forming units, [cfu]/g), but they rapidly dominate the fermentation due to favorable conditions (Rantsiou and Cocolin 2006). In addition to their ability to produce lactic acid and to reduce pH, LAB are also competitive in vegetable and meat fermentations because of their ability to produce

bacteriocins. Bacteriocins are small peptides (30–60 aa) with antimicrobial properties against bacteria usually of the same or closely related species (narrow spectrum), and occasionally against a broader spectrum of species (Klaenhammer 1988; Rantsiou et al. 2006; Galvez et al. 2008; Settanni and Corsetti 2008, see also Chapter 5). The approach taken traditionally to study bacteriocin production has dramatically changed with the advent of genome sequencing. One example is the comparative genomics approach used to identify bacteriocin-like genes in the Lactobacillales (Makarova and Koonin 2007). Since the gene sequences of the bacteriocin determinants are highly divergent, Makarova and Koonin (2007) identified genes that are commonly found clustered and associated to bacteriocin-coding sequences. Using this approach they were able to identify such clusters in seven *Lactobacillus* genomes. The candidate bacteriocins were homologs of pediocin (Fimland et al. 2005) and divercin V41, a class IIa bacteriocin naturally produced by *Carnobacterium divergens* V41 and similar to pediocins and enterocins (Rihakova et al. 2008).

### 2.2.3. Fast-Evolving LAB

Wines that undergo good malolactic fermentation are often described as “fuller,” more complex, and less acidic, sometimes with “buttery” overtones. It is well known that LAB are responsible for the reduction in the acidity of wine by the conversion of malic acid into lactic acid and CO<sub>2</sub>. The LAB isolated from the wine environment (Pilone et al. 1966; du Plessis et al. 2004; Bae et al. 2006) mostly belong to the genera *Pediococcus*, *Lactobacillus*, *Leuconostoc*, and *Oenococcus*. Availability of the genome sequence of *Oenococcus oeni* PSU-1 was the starting point for genetic and metabolic studies as well as comparative genomics analyses (Mills et al. 2005). Recently, Marcobal et al. (2008) analyzed the rate of spontaneous mutations for rifampin and erythromycin resistance and demonstrated that *O. oeni* PSU-1 and the other species in the genus *Oenococcus* *kitaharae* NRIC0645 exhibit a higher spontaneous mutation rate compared with their phylogenetic neighbors, *Leuconostoc mesenteroides*

ATCC8293 and *Pediococcus pentosaceus* ATCC25745. The main difference between the strains of *Oenococcus* and the other strains was that the latter contain *mutS* and *mutL* genes (Makarova et al. 2006), which encode two key enzymes involved in DNA mismatch repair. MutS binds to the DNA mismatch; MutL joins the MutS–DNA complex and activates MutH, which is an endonuclease that cuts the non-methylated strand within a hemimethylated GATC sequence (Stanislawska-Sachadyn and Sachadyn 2005). The consequences of absence of *mutS* and *mutL* in *O. oeni* is of technological relevance since this is the most rapidly evolving species of the LAB and the organoleptic properties of wine that has undergone malolactic fermentation are usually highly strain specific (de Las Rivas et al. 2004).

### 2.2.4. Genetic Make-Up of LAB in the GI Tract

The presence of LAB in the GI tract of animals and humans is widely recognized and has been reviewed, both before (Salminen and Deighton 1992) and after (Walter 2008) the explosion of genome sequencing projects. In the GI tract system, determination of the individual components is a complex effort as the GI tract provides an anaerobic environment rich in enzymatic activities, bile, and extreme pH conditions (Sanderson 1999). As a consequence, the human microbiome is composed of a vast number of noncultivable bacterial species compared with only a fraction that can be cultured and manipulated in a laboratory. The Human Microbiome Project (HMP; <http://nihroadmap.nih.gov/hmp/>) initiative has begun to elucidate the fascinating symbiotic relationship between microbial commensal organisms and their human hosts. Our microbiota allow us to harvest otherwise inaccessible nutrients and is an intrinsic part of our metabolism. Presently, there are >600,000 bacterial ribosomal DNA sequences in GenBank; over 15,000 are annotated as derived from the human GI tract; and >6000 annotations represent uncultured bacteria from the phyla *Firmicutes* and *Bacteroidetes*. With the characterization of the core human microbiome under way,

identification of these important taxonomic groups in existing human samples will be a key contribution to the HMP. Furthermore, the identification of a potential relationship between genetics, lifestyle, and the human probiome, defined as commensal intestinal bacteria considered to have a beneficial influence on human health (Azcarate-Peril et al. 2008), will begin to unravel the complex host-microbiome equilibrium. The ultimate objective of the HMP is to link members of the human microbiome to states of health or disease, such as obesity-related diabetes.

The human body maintains a relationship that varies from parasitic to beneficial with bacterial numbers ranging from  $10^1$  to  $10^3$  cfu/ml in the stomach,  $10^4$  to  $10^7$  cfu/ml in the distal small intestine, and a maximum of  $10^{11}$  to  $10^{12}$  cfu/ml in the colon (O'Hara and Shanahan 2006). Human microbial communities are overwhelmingly dominated by four phyla: Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria. The relative abundance of these phyla tends to be consistent across individuals (Dethlefsen et al. 2007). In particular, representation of *Lactobacillus* sequences in molecular-phylogenetic analysis of the human gut microbiota varies from 0.03% to 9.9% depending on the type of methodology and analysis used, the number of sequences, and the sampling area (Walter 2008). The majority of *Lactobacillus* species found in the GI tract include *Lactobacillus mali*, *Lactobacillus reuteri*, *Lactobacillus delbrueckii*, *Lactobacillus rhamnosus*, and *Lactobacillus animalis*, according to (Table 2.3; Walter 2008) and *Lactobacillus gasseri*, *Lact. reuteri*, *Lactobacillus salivarius*, *Lactobacillus casei*, *Lactobacillus plantarum*, and *Lactobacillus buchneri*, according to Reuter (2001). At present, a number of probiotic bacteria of human origin are exploited commercially, including *Lact. casei* Shirota (Morishita et al. 1974), *Lact. acidophilus* NCFM (Gilliland et al. 1975), *Lact. rhamnosus* GG (Silva et al. 1987), and *Lact. acidophilus* LA-1 (Bernet et al. 1994).

A multi-genome homology comparison between available LAB genomes at The Institute for Genomic Research (TIGR) database (<http://cmr.jcvi.org/tigscripts/CMR/CmrHomePage.cgi>) and a reference

genome sequence were performed (see Fig. 2.2 in the color plate section; Peterson et al. 2001). For this comparison *Clostridium difficile* QCD-32g58 (NAP1/027), isolated from human stools during a *C. difficile* outbreak in 2003 and 2004 in Québec, Canada was chosen (Loo et al. 2005). As expected, the analysis identified another stool isolate, *Enterococcus faecalis* V583 as the organism showing the highest number of protein matches (137) to the reference versus the probiotic *Lact. acidophilus* NCFM showing the lowest number of matches (50). Matches between *Ent. faecalis* and *C. difficile* included a protein annotated as a pili retraction protein PilT in the reference strain. PilT is part of the type IV pili complex, a motor protein that catalyzes pilus retraction. Type IV pili are multifunctional complexes that can act as virulence factors (for motility); they are also used to build multicellular structures like biofilms (Varga et al. 2008). Other high-conserved matches include stress-related proteins (GroEL, oxidoreductases, Clp proteins, ethanolamine utilization proteins), transporter proteins (amino acid and ATP-binding cassette transporters), NTP hydrolases (TraG-like) involved in DNA transfer in bacterial conjugation (Cabezon et al. 1997), and a number of unknown conserved proteins. Twenty-six reference proteins were present in all comparison molecules at a 60% identity cutoff, mostly ribosomal proteins (17), and also *metK*, *upp*, and the alpha and beta subunits of the ATP synthase complex. These analyses suggested that despite sharing the same intestinal environments, nonpathogenic LAB have a significantly different genetic makeup. Genome homology analysis using *Lact. acidophilus* NCFM as the reference organism, versus all available genome sequences at TIGR (total protein hits, including multiple hits per protein), exhibited highest homologies between phylogenetic groups of lactobacilli (*Lact. johnsonii* and *Lact. plantarum*) and bacilli (see Fig. 2.3 in the color plate section). *Clostridium beijerinckii* NCIMB 8052 (GeneBank number CP000721), a nonpathogenic butanol-producer organism, also showed high levels of homology, clustering with the bacilli group and not the rest of clostridia strains.

**Table 2.3.** Representation of *Lactobacillus* sequences in molecular-phylogenetic analysis of human gastrointestinal microbiota (Walter 2008). Reproduced with permission.

Reported sample site(s) or material	No. of subjects	Total no. of sequences	No. of <i>Lactobacillus</i> sequences	% of <i>Lactobacillus</i> sequences	Reference
Stomach tissue	23	1833	4	0.22	Bik et al. (2006)
Small intestine tissue, non-IBD	20	1638	5	0.31	Frank et al. (2007)
Jejunum, ileum tissue	1	173	0	<0.6	Wang et al. (2005)
Jejunum and ileal lumen	3	545	87 <sup>a</sup>	16	Hayashi et al. (2005)
Ileal and colon tissue	2	361	0	<0.3	Wang et al. (2003)
Colon and rectal tissue	1	174	0	<0.6	Wang et al. (2005)
Colon and rectal lumen	3	545	54 <sup>b</sup>	9.9	Hayashi et al. (2005)
Cecal, colon, and rectal tissue and feces	3	11,831	0	<0.01	Eckburg et al. (2005)
Colon tissue, non-IBD	40	3214	157 <sup>c</sup>	4.9	Frank et al. (2007)
Colon tissue	3	110	0	<1	Eckburg et al. (2005)
Feces	4	927	0	<0.11	Hayashi et al. (2002a, 2002b)
Feces	1	284	0	<0.4	Suau et al. (1999)
Feces	12	18,348	6 <sup>d</sup>	0.03	Ley et al. (2006)

<sup>a</sup>The species detected were *Lactobacillus mali* (85 sequences) and *Lact. reuteri* (2 sequences).

<sup>b</sup>The species detected were *Lactobacillus reuteri* (27 sequences), *Lact. mali* (20 sequences), and *Lactobacillus delbrueckii* (7 sequences).

<sup>c</sup>The main species detected were *Lact. delbrueckii* (108 sequences), *Lactococcus rhamnosus* (38 sequences), *Lact. reuteri*, and *Lactobacillus animalis* (each 5 sequences).

<sup>d</sup>Sequence identification was performed using the Classifier tool of the Ribosomal Database Project II (108) with a confidence threshold of 80%; the complete sequence data set was kindly provided by Ruth Ley (Washington University, St. Louis, MO).

### 2.3. The Impact of Genome Sequencing on the Taxonomy of LAB

The explosion of available genomic information has impacted our phylogenetic view of the traditional concept of “species.” The most accepted definition of species today is the phylo-phenetic concept: “a species is considered a monophyletic and genomically coherent cluster of individual organisms that show a high degree of overall similarity with respect to many independent characteristics, and is diagnosable by a discriminative phenotypic property” (Rossello-Mora and Amann 2001; Felis and Dellaglio 2007). Prior to the genome sequencing boom, the criteria used to define “species,” which is the essential entity of biological diversity, included phenotypic (morphological and physiological characteristics, cell wall composition, protein fingerprinting, electrophoretic mobility of enzymes, fatty acid methyl ester analysis) as well as genotypic analysis (GC content analysis, DNA-DNA and DNA-

RNA hybridization, ribotyping, DGGE, RAPD-PCR, multiple locus sequence typing) (Ludwig and Schleifer 1994; Vandamme et al. 1996; Klein et al. 1998; Carr et al. 2002; Felis and Dellaglio 2007).

With the beginning of the genome sequencing era, comparative genomics studies confirmed the notion that bacterial species share a pool of common genes, the core genome or pangenome. The slow divergence from the core constitutes one of the main sources of variability among genomes. Recently, Makarova and Koonin (2007) defined the core genome of 12 sequenced genomes of the group Lactobacillales, which share 567 Clusters of Ortholog Groups. The majority of genes included in the core were involved in replication, transcription, and translation, but approximately 100 genes were poorly characterized. One interesting observation was the presence of two core genes with no detectable orthologs outside Lactobacillales. One of these unique genes encodes a protein containing a peptidoglycan-binding LysM domain (LaCOG01826)



and the second (LaCOG01237) contains no characterized domains but is located in a conserved genomic region, which also includes two enzymes implicated in 4-thiouridine modification of tRNA ([5-methylaminomethyl-2-thiouridylate] methyltransferase and a predicted sulfurase; LaCOG00578 and LaCOG01188), suggesting a related role.

Before whole genome sequences of multiple strains from the same species were available, comparative genome hybridization (CGH) was employed to compare strains of *L. lactis* (Kok et al. 2005). DNA microarray hybridizations of *L. lactis* IL1403 with a mixture of randomly Cy3- or Cy5-labeled DNA fragments from the *L. lactis* IL1403 and *L. lactis* MG1363 showed a positive correlation between gene similarity and the obtained ratio for genes with a similarity index of >75%. The study also highlighted genes present in IL1403 but not in MG1363 (*glpD*, *glpK*, *dexB*, *yabE*, *rnaF*, *lcnC*, *umuC*, and *pi251*). More recently, analysis of the genome sequence of MG1363 (Wegmann et al. 2007) revealed approximately 85% DNA sequence identity between coding sequences (CDS) present in both *L. lactis* MG1363 and *L. lactis* IL1403 and 97.7% between those in the two subspecies *cremoris* strains, *L. lactis* MG1363 and *L. lactis* SK11 (Makarova et al. 2006). Additionally, a core genome containing approximately 58% of coding sequences was also defined for *Streptococcus thermophilus* (Rasmussen et al. 2008). The authors selected 2200 relevant CDS from available *Strep. thermophilus* genomes (Bolotin et al. 2004; Makarova et al. 2006) as input to design 65- to 75-mer oligonucleotides for a microarray platform. CGH of 47 dairy *Strep. thermophilus* strains identified 1271 core genes and confirmed the absence of virulence genes.

Undoubtedly, the extensive characterization of bacterial strains at genomic resolution reveals important genetic differences between strains and will contribute to a more stringent definition of “species.” A recent study by Deloger et al. (2009) points out that a second source of variability among genomes, beyond the divergence of the core genome, is due to the rapid gain and loss of large DNA portions, especially within species. From this concept, the authors identify different approaches to estimate

genome distances. The “Average Nucleotide Identity” (ANI) is “the average nucleotide identity of the total genomic sequence shared between two strains” (Konstantinidis et al. 2006). This method defines the list of orthologs and derives the overall divergence of the core genome by averaging the percentage of identity at the nucleotide level of all orthologs found. Other methods approximate distances based on estimating the proportion of common genes or DNA. The approach proposed by Deloger et al. (2009) intends to capture both dimensions of genome variability at the inter- and intraspecies levels based on the amount of Maximal Unique and Exact Matches (MUMs) of a given minimal length shared by the compared genomes. A MUM index (MUMi) analysis of 67 genera for which at least two genomes are available indicated relative homogeneity within species, and also showed a correlation between MUMi, ANI, and the more classical method, Multi Locus Sequence Typing analysis. The MUMi for *L. lactis* subspecies *lactis* versus *L. lactis* subspecies *cremoris* was 0.74 indicating high diversity (the MUMi varies between 0, for very similar, and 1, for very distant genomes) at the subspecies level. This analysis correlates well with other observations of high intraspecies variability in LAB. For example, phenotypic analysis of nine strains of *Lact. gasseri* of intestinal origin revealed high intraspecies variability. This level of variability highlights the importance of strain sequencing and in-depth studies of strain-specific genetic systems (Azcarate-Peril et al. 2008). Another example was observed within *C. divergens*, a ubiquitous LAB frequently isolated from meat, fish, shrimp, and some dairy products at low temperatures (Leisner et al. 2007). A study by Laursen et al. (2005) used numerical taxonomy based on classical biochemical reactions, carbohydrate fermentation and inhibition tests (temperature, salt, pH, chemical preservatives, antibiotics, bacteriocins), SDS-PAGE electrophoresis of whole cell proteins, plasmid profiling, intergenic spacer region (ISR) analysis, and examination of amplified-fragment length polymorphism to characterize over 100 *Carnobacterium* strains (50 from beef and pork products, and 52 from cod, halibut, salmon, shrimps, and roe products). Interestingly,

the approach taken by the investigators divided the carnobacteria strains into 24 groups that shared less than 89% similarity. These groups were identified mainly as *C. divergens* and *Carnobacterium maltaromaticum* and both species exhibited high phenotypic intraspecies variability although a reliable identification was obtained by SDS-PAGE of whole cell proteins and by ISR based on 16S-23S rDNA intergenic spacer region polymorphism. Presently, no strains of *C. divergens* are being sequenced; however, the genome of *Carnobacterium* sp. AT7 (Accession number NZ\_ABHH00000000), a piezophilic strain isolated from the Aleutian trench at a depth of 2500 m, is being determined.

## 2.4. A "Probiotic Island"

Considerable discussion followed genomic studies of organisms considered true autochthonous species of the human intestinal probiome (Azcarate-Peril et al. 2008). According to Reuter (2001), *Lact. gasseri* and *Lact. reuteri* are the *Lactobacillus* species native to the human GI tract. True transient lactobacilli include *Lact. salivarius*, *Lact. plantarum*, *Lact. casei*, *Lact. buchneri*, *Lactobacillus brevis*, and *Lactobacillus fermentum*. However, a more recent review concludes that most *Lactobacillus* species found in the mammalian GI tract are not true intestinal inhabitants but they originate from proximal or exogenous sources (Walter 2008). Interestingly, a number of factors allow probiotic lactobacilli to persist in the gut, and some of those factors are also considered contributors to bacterial "virulence" indicating, not surprisingly, that both probiotic and pathogenic bacteria can use similar strategies to survive in the GI tract. The concept of a "probiotic island" as a genomic region encompassing genes involved in probiotic functionalities arise as a counterpart of the "pathogenicity island" described in virulent species such as *Ent. faecalis* (Coburn et al. 2007) and *Salmonella enterica* (Halici et al. 2008). One example of such probiotic islands originated from the analysis of the complete genome sequences of the reuterin-producing *Lact. reuteri* JCM 1112<sup>T</sup> and its closely related species *Lact. fer-*

*mentum* IFO 3956 (Morita et al. 2008). Comparative genome analysis revealed that JCM 1112<sup>T</sup> has a unique cluster of 58 genes for the biosynthesis of reuterin and cobalamin (Taranto et al. 2003), which has a lower GC content and is apparently inserted into a conserved region, resulting from a horizontal gene transfer event (see Fig. 2.4 in the color plate section). Production of 3-hydroxypropionaldehyde from glycerol by *Lact. reuteri* was first reported by Talarico and Dobrogosz (1989). The authors characterized this substance, termed reuterin, and showed the inhibited growth of Gram (+) and Gram (–) bacteria as well as yeasts, fungi, and protozoa. Synthesis of reuterin is mediated by glycerol dehydratase (E.C. 4.2.1.30). According to Morita et al. (2008) the genome of JCM 1112<sup>T</sup> contains three genes (LAR\_1633-1635) with dehydratase subunit motifs (Pfam PF02286-02288) in the propanediol utilizing operon (LAR\_1616-1640). The genes have been designated *gupCDE* (glycerol utilization gene candidates in the *pdu* operon) based on homology with other bacterial strains. The encoded amino acid sequences exhibited the highest identity to their homologs from *Lact. brevis* ATCC 367 (Makarova et al. 2006), showing 81%, 66%, and 57% identities, respectively. Additionally, a cluster responsible for cobalamin biosynthesis (*cbi*, *cob*, and *hem*) has been identified and located adjacent to the *pdu* operon. Notably, the organization of this genomic island represents a close association of the *pdu* and *cbi-cob* operons reflecting the cobalamin requirement for glycerol dehydratase activity. A comparison of *pdu* and *cbi-cob* operons from *Lact. reuteri* with *Lact. fermentum* IFO 3956 (Morita et al. 2008) and *Lact. plantarum* WCFS1 (Kleerebezem et al. 2003) revealed not only that the *pdu-cbi-cob-hem* gene cluster comprising 58 open reading frames (ORFs; LAR\_1583-1640) was absent in these LAB but also that it was inserted in a locus that was common to all three bacteria (Fig. 2.4). Interestingly, a comparison with the draft genome sequence of *Lact. reuteri* 100-23, a rodent-specific strain, showed that the *pdu-cbi-cob-hem* gene cluster is absent. We can foresee more examples of probiotic islands as strain sequencing becomes more widespread and affordable.

## 2.5. Other Probiotic Features

A number of probiotic features have been identified in LAB, although most of them are not as manifestly organized as the *pdu-cbi-cob-hem* island from *Lact. reuteri*. The majority of the probiotic features are related with the survival of the strain in the GI tract. The intestinal environment results from three main factors: dietary intake, bacterial ecology, and host physiology, including factors such as peristalsis and glandular secretions. Several factors restrict bacterial cell growth including gastric acidity, oxidative stress, digestive enzymes, bile salts, peristalsis, mucus, the resident commensal microflora, exfoliation of enterocytes during epithelial renewal, epithelial translocation of secretory IgA, CD8<sup>+</sup> intraepithelial T lymphocytes, and innate host defense mechanisms mediated by gene-encoded antimicrobial peptides (Sanderson 1999; Ouellette 2004). LAB are typically regarded as aerotolerant anaerobes (Axelsson 1998) that can grow in the presence of oxygen and generate partially reduced reactive oxygen species (ROS) including the superoxide radical (O<sub>2</sub><sup>-</sup>), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), and the hydroxyl radical (HO). ROS are both cytotoxic and mutagenic and, in the GI tract, ROS are central players in inflammatory bowel disease (IBD; Sartor 2004). To atone for the harmful effects of ROS, both bacterial and intestinal cells have evolved protective mechanisms that utilize antioxidant enzymes such as superoxide dismutases (SODs) and hydroperoxidases to prevent the formation of HO. Since the levels of SOD in IBD patients are frequently depleted (Lih-Brody et al. 1996), there is potential for elevated levels of this enzyme to function therapeutically for IBD. LAB-encoding genes involved in both oxidative stress resistance and regulatory mechanisms have been identified (Miyoshi et al. 2003; El-Sharoud 2005) and oxidative resistance genes, such as SOD and catalase, have been expressed in probiotic strains such as *Lact. gasseri* (Bruno-Barcena et al. 2004; Carroll et al. 2007) and technologically relevant organisms like *Lact. delbrueckii* subsp. *bulgaricus* (Rochat et al. 2006).

One important attribute of probiotic LAB is their ability to resist and thrive in acidic environments (Kullen and Klaenhammer 1999; Gueimonde and Salminen 2006) like the stomach of mammals and fermented low-pH foods. Several mechanisms of acid resistance have been described in LAB. The most important and universally present in the group, the multisubunit F<sub>1</sub>F<sub>0</sub> ATPase, which links the production of ATP molecules to the transmembrane proton motive force (PMF), can either generate ATP at the expense of PMF or produce PMF-consuming ATP. The PMF facilitates the extrusion of protons from the cytoplasm. The F<sub>0</sub> complex has proton translocating activity, while the peripherally bound F<sub>1</sub> complex has ATPase activity (Nath 2002). Another mechanism of acid resistance is the Arginine Deiminase (ADI; E.C. 3.5.3.6) pathway. Present only in a limited number of members of the LAB group (Arena et al. 2002; Cotter and Hill 2003), this enzyme also exhibits anticarcinogenic activities. A recent study investigated the anti-inflammatory activities of the purified recombinant ADI from *L. lactis* subsp. *lactis* ATCC7962 (Kim et al. 2007b), and showed that ADI strongly inhibits the generation of nitric oxide (NO) and PGE2 in LPS-treated macrophages by inhibiting expression of the inducible NO synthase (iNOS) and cyclooxygenase (COX-2) proteins. The purified enzyme also inhibited the production of IL-6 in LPS-treated RAW 264.7 macrophages. The enzyme responsible for the overproduction of NO, iNOS, is often observed during inflammation and tumor development.

Other acid resistance mechanisms include urease, a nickel containing oligomeric enzyme that catalyzes the hydrolysis of urea to two molecules of ammonia and one molecule of carbon dioxide (Mobley et al. 1995). The enzyme has maximal activity at pH 7 but loses activity at more extreme acidic pHs, being essentially inactive at pH 4.3. Ureases have been mostly associated with pathogens with the most studied system encoded by the highly urolytic bacteria *Helicobacter pylori* (Bartnik 2008). However, urease homologs have been identified in *Lact. fermentum* (Kakimoto et al. 1990), *Lact. brevis* ATCC 367 (LVIS\_1854), and *Leuconostoc mesenteroides*

subsp. *mesenteroides* ATCC 8293 (LEUM\_2023). Interestingly, *Strep. thermophilus* is the only member of the food-grade LAB to encode a functional urease (Makarova et al. 2006).

Amino acid decarboxylases are also involved in acid resistance. These enzymes process the bond holding the carboxylic (-COOH) group to the rest of the amino acid. As a result, the end-product is a basic chemical that causes the pH to increase. The glutamate decarboxylase (Gad) system of acid resistance has been extensively characterized in Gram (-) and Gram (+) bacteria (Sanders et al. 1998; Waterman and Small 2003; Tramonti et al. 2006). In *L. lactis*, expression of *gadCB* in the presence of chloride increases in modified M17 medium, in which  $\beta$ -glycerophosphate was omitted to lower the buffering capacity of the medium, when the culture pH decreases. Expression of *gadCB* is also induced by glutamate (Sanders et al. 1998). Glutamate decarboxylase homologs were identified in a limited number of LAB besides *L. lactis*, including *Lact. plantarum* WCFS (lp\_3420) and *Lact. brevis* ATCC 367 (LVIS\_0079, LVIS\_1847, and LVIS\_2213). However, the transport component of the Gad system (GadC) appears to be widely distributed in LAB. In *Lact. acidophilus* NCFM, GadC is one of the mechanisms responsible for the intrinsic acid resistance of the strain (Azcarate-Peril et al. 2004).

Another mechanism that has received attention recently involves the density-dependant recognition of an autoinducer molecule by bacterial cells with consequent changes in gene expression. Several studies have reviewed the role of this mechanism, termed *quorum sensing*, in human health (Jayaraman and Wood 2008; Sifri 2008; Willcox et al. 2008), food fermentation (Fleet 2003; Sieuwerts et al. 2008), food spoilage (Ammor et al. 2008), and production of antimicrobial molecules (van der Ploeg 2005; Petersen et al. 2006; Straume et al. 2007; Navarro et al. 2008). One important *quorum sensing* system used to communicate among and between species is based on a furanosyl borate diester called autoinducer-2 (AI-2), produced in four enzymatic steps from methionine. The gene encoding the AI-2 synthase, *luxS*, has been identified in many different

Gram (+) and Gram (-) species (Vendeville et al. 2005; Lebeer et al. 2007; Buck et al. 2009). AI-2 regulates the expression of various phenotypes including virulence factors, DNA processing, cell morphology, motility, biofilm formation, toxin production, light production, cell division, and adhesion (Xavier and Bassler 2003; Lebeer et al. 2007; Sztajer et al. 2007; Buck et al. 2009).

Highly conserved homologs of *luxS* are present in over 55 species of Gram (-) and Gram (+) bacteria (Kaper and Sperandio 2005). Figure 2.5 (see in the color plate section) shows a comparison of the region containing *luxS* in several LAB genomes that exhibit over 70% similarity levels in this gene. As previously reported (Buck et al. 2009), it is notable that despite the high degree of similarity of *luxS*, different species show markedly different organizations in the surrounding genomic regions. Furthermore, *L. delbrueckii* subsp. *bulgaricus* ATCC BAA-365 (Makarova et al. 2006) encodes two copies of *luxS* in distant loci (LBUL\_0088 and LBUL\_1231) with no apparent gene synteny.

## 2.6. Functional Genomics Studies to Unveil Novel LAB Utilities

The presence of a large number of “ORFans,” which are defined as annotated genes that are restricted to a particular genome and that possess no known homologs (Daubin and Ochman 2004), has been observed widely in a number of genome and comparative genome papers. A MUMmer plot of the chromosome *Strep. thermophilus* CNRZ1066 versus the chromosome of *Strep. thermophilus* LMG 18311 (see Fig. 2.6 in the color plate section) shows very high similarity levels; however, several genes can be identified as unique for each one of the analyzed strains (represented as red and green dots). The uniqueness of ORFans within a genome has not allowed the use of classic comparative approaches to determine their function and evolution. While other bioinformatic methods can provide a theoretical guide for the functionality of ORFan genes, only functional genomics approaches can confirm that function to potentially uncover novel LAB features.



Numerous studies have demonstrated the importance of functional genomics in LAB research. In 2002, Jansen et al. (2002) identified a novel family of repetitive DNA sequences, present among both domains of the prokaryotes (Archaea and Bacteria), but absent from eukaryotes or viruses. The family, identified by *in silico* analysis, was characterized by direct repeats from 21 to 37 bp, interspaced by similarly sized non-repetitive sequences. This family was termed Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR; Jansen et al. 2002). As genome sequencing projects progressed CRISPRs were identified in an increasing number of bacterial genomes; however, their function in phage resistance was only elucidated in 2007 (Barrangou et al. 2007) by altering the CRISPR locus of a dairy strain of *Strep. thermophilus* DGCC7710. Through adding and deleting spacers, derived isogenic strains were shown to be sensitive or resistant to two virulent bacteriophages isolated from industrial yogurt samples, phage 858 and phage 2972 (Levesque et al. 2005).

Another example of *in silico* predictions of metabolic functions that were confirmed by functional genomics analyses is provided by the pathway-genome database of *L. plantarum* WCFS1, LacplantCyc (<http://www.lacplantcyc.nl/>; Teusink et al. 2005). LacplantCyc was automatically reconstructed using the Pathologic tool of Pathway Tools (<http://bioinformatics.ai.sri.com/ptools/>) and manually curated. The vitamin and amino acid requirements of *Lact. plantarum* were evaluated experimentally in single-omission assays where each of the vitamins and amino acids was separately left out of the medium. In the majority of cases (32 of 37 cases), the experimental results agreed with the final reconstruction. In the case of the B vitamins, folic acid (vitamin B11), thiamine (vitamin B1), and pyridoxine/pyridoxamine (vitamin B6), growth experiments showed that addition to the medium was not required, indicating that the pathways related to the biosynthesis of these compounds should be complete and active. However, the pathways seemed incomplete and the gaps had to be filled by combining sequence and published information from other organisms. The opposite occurred

with amino acid requirements. The genome sequence of *Lact. plantarum* revealed that only the pathways for the biosynthesis of the branched-chain amino acids Ile, Leu, and Val were absent. However, three other amino acids (arginine, glutamate, and tryptophan) could not be removed from the media indicating an auxotrophy for these amino acids. In the case of arginine, the authors propose cumulative mutations in the genes involved in arginine biosynthesis to be responsible for the auxotrophy. The other two amino acid auxotrophies revealed erroneous gene annotations.

The development of molecular tools like vectors and systems to insertionally inactivate, delete, or overexpress specific loci in the genome has been invaluable in the definition of gene function and creation of recombinant LAB. Genetic manipulations systems were developed earlier for *L. lactis*; natural gene transfer systems (transduction, conjugation, transposition) were routinely employed over 20 years ago (Fitzgerald and Gasson 1988). Later, a gene replacement vector based on the *Escherichia coli* vector pMTL23p with a *Cm<sup>r</sup>* gene from pC194 (Iordanescu and Surdeanu 1980) and a *nisA* gene from pFI172 (Dodd et al. 1990) inactivated by insertion of an *Em<sup>r</sup>* gene from pE194 was constructed for insertional inactivation of the nisin production gene *nisA* (Dodd et al. 1992). Although some early attempts of genetic manipulation in lactobacilli were successful (O'Sullivan and Klaenhammer 1993; Bhowmik and Steele 1994; Skaugen and Nes 1994), only a restricted number of strains were susceptible to such manipulation and the developed tools achieved limited success. More recently, systems for insertional inactivation and heterologous expression of interesting genes were developed based on available genomic information to support functional genomics studies (Russell and Klaenhammer 2001; Bruno-Barcena et al. 2005; Klaenhammer et al. 2005; Lambert et al. 2007). The available expression and secretion systems for heterologous protein secretion in *L. lactis*, including promoters, signal peptides, and mutant host strains, have been reviewed recently by Morello et al. (2008). Earlier, functional studies that allowed the identification of gene function based on genomic information in

lactobacilli was reviewed by Claesson et al. (2007) and Klaenhammer et al. (2005).

One of the most exciting promises of LAB is the prospect of using Generally Recognized as Safe members of this group as delivery vehicles for vaccines, antimicrobial compounds, and therapeutics. An early study demonstrated that intragastric administration of a recombinant *L. lactis* strain, secreting murine IL-10, prevented onset of colitis in IL-10 knockout mice, and caused a 50% reduction of the inflammation in dextran sulfate sodium-induced chronic colitis (Steidler et al. 2000). In 2006, the first pilot trial with a recombinant LAB in humans was published (Braat et al. 2006). The authors replaced the *thyA* gene of *L. lactis* with a synthetic sequence encoding mature human IL-10, fused at its N-terminus to a lactococcal secretion signal. They chose this method because it provided an adequate containment for the recombinant bacteria since the modified strain of *L. lactis* was unable to survive in the environment without thymidine or thymine. The results of this study were very promising as clinical benefit was observed in 8 of 10 patients. A recent article by Wells and Mercenier (2008) reviewed the accumulated existing scientific data on the use of LAB as mucosal delivery vehicles of vaccine antigens, microbicides, and therapeutics (for another revision see this book, Chapter 9). Current applications of LAB delivery include the production and delivery of single-chain variable fragments, DNA, peptides, antigens, cytokines, enzymes, and allergens. The only study using a human model involved the delivery of cytokines to determine the effect on IBD and colitis.

## 2.7. Conclusions

With 779 complete microbial genomes and 1310 microbial genomes in progress at NCBI, comparative genomics has revealed complex evolutionary relationships within specific bacterial phylogenetic groups and also has answered essential evolutionary questions. In particular, LAB genomics has contributed significantly to our understanding of the relationships between genotype and phenotype, which will have important ramifications in controlling bio-

technologically relevant processes, in developing novel vaccines, and in improving diagnostics. Furthermore, we can envision that strain sequencing will become routine with the development of next generation, massively parallel ultra-high throughput sequencing methods like Solexa (Illumina), SOLiD (Applied Biosystems), 454 (Roche) Heliscope (Helicos BioSciences Corp.), and Polonator G.007 (Danaher Motion and George Church). These platforms support a wide range of genetic analysis applications from reference assembly of genomes, resequencing, *de novo* assembly, SNP detection, digital gene expression, metagenomics, genomics and transcriptomics analysis (Shendure and Ji 2008), and downstream applications like polymerase colony (polony) multiplex analysis of gene expression (PMAGE; Kim et al. 2007a). PMAGE is an adaptation of serial analysis of gene expression and polony sequencing, where RNA is converted to cDNA, and specialized restriction endonucleases are used to cleave a short sequence tag of about 14 bp from each transcript. Individual transcript tags are then clonally amplified on beads in millions of parallel compartmentalized droplets in a water-in-oil emulsion. The amplified beads are placed on a glass surface, and the identity of the tags is inferred by sequencing by ligation. DNA ligase labels each bead with a fluorescent label corresponding to the identity of a base within the template and microscopic approaches detect each fluorescence ligation event. Parallel sequencing and enumeration of the tags is finally used to calculate the abundances of the corresponding transcripts. Given the vast amounts of data generated by these methods, High Performance Computing systems to analyze, visualize, cluster, and store data have driven development of increasing numbers of bioinformatic tools and databases (Altermann and Klaenhammer 2005; Teusink et al. 2005; Kiefer et al. 2008; Pons et al. 2008; Waagmeester et al. 2008). Bioinformatics may remain the bottleneck in the elucidation of important pathways in LAB. However, functional genomics studies, in a high-throughput fashion, will be the pillar to unveil novel functionalities in these microbes that are so beneficial in food preservation, bioprocessing, and health.



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## Chapter 3

# Proteomics: A Tool for Understanding Lactic Acid Bacteria Adaptation to Stressful Environments

Marie-Christine Champomier-Vergès, Monique Zagorec, and Silvina Fadda

*The rapid expansion of proteomics built upon the available bacterial genome sequences has provided new approaches for bacterial functional genomics. In combination with transcriptional profiling expression, proteomics provides access to interesting candidate genes and proteins that can be further characterized by traditional, physiological and biochemical, and genetic analyses. The action of multiple gene sets can now be revealed by the combined technologies of genomics, proteomics, and bioinformatics, which constitute valuable tools to understand these complex phenomena. Insights on different proteomic aspects from theoretical and technical topics up to applied microbial case studies are described in this chapter. Recent developments related to bacterial adaptation to the environment and the definitions of technological determinants at the proteome level for economically important lactic acid bacteria, involved mainly in food fermentation, are discussed.*

### 3.1. Introduction

Fermentation and drying can be considered the oldest ways of preserving raw materials by extending the shelf life as well as enhancing the flavor and nutritional qualities of the products. Lactic acid bacteria (LAB) are the main agents responsible for fermentation. These bacteria reduce the ripening time, minimize the manufacturing defects, improve the sensory properties, and inhibit the development of pathogenic and spoilage flora (Liepe 1983).

Moreover, LAB are also considered the most important microorganisms responsible for the health-promoting effects of fermented foods, specially in milk-derived products.

#### 3.1.1. Effects of LAB on Sensory Properties

The final consistency of fermented products such as cheese or sausages is the result of their acidification and dehydration. The fermentation of carbohydrates by LAB mainly leads to the formation of lactic acid, particularly D-lactate, which reduces the pH to about 5.0 and causes protein coagulation contributing to the firmness of the final product. Acidification also reduces the water retention capacity of the mixture favoring the dehydration of the product and, in some fermented foods such as cured meat products, indirectly contributes to color development. Flavor, which includes taste, aroma, and odor, is one of the most sought properties in foods and its perception depends on the texture of the product. It is the result of a careful balance between volatile and nonvolatile compounds; these coming from the catabolism of carbohydrates, proteins, and lipids from tissue and microbial enzyme activities and chemical reactions, lactate conferring the typically acid taste of fermented foods. Heterofermentative LAB catabolize glucose and pentoses to lactic acid, acetic acid, and ethanol. Ethanol does not contribute directly to flavor but it can be involved in secondary reactions resulting in highly odorous ethyl esters conferring a fruity note on the product. The buttery or

dairy aroma characteristic is related to diacetyl and acetoin, which result from the metabolism of pyruvate (Molimard and Spinnler 1996; Talon et al. 2002). Proteolysis is carried out by LAB and other enzymes from the food matrix; the resulting free peptides and amino acids improve flavor and the nutritional value of the fermented foods (Smit et al. 2005). For specific flavor development, further conversion of amino acids to various alcohols, aldehydes, acids, esters, and sulfur compounds is required. LAB in general scarcely contribute to lipolysis, but adjuvants cultures (e.g., molds or *Staphylococcus* and *Kocuria* in the case of surface-ripened cheeses or sausages, respectively) often display high activities in fat conversion (Molimard and Spinnler 1996; Talon et al. 2002), the released free fatty acids being precursors of flavor compounds (methylketones, secondary alcohols, esters, and lactones).

### 3.1.2. Effects of LAB on Hygienic Properties

Certain LAB strains inhibit the pathogenic and spoilage flora present in food. The inhibiting properties of LAB are attributed mainly to the production of organic acids, particularly lactic acid and acetic acid, which are responsible for the decrease in pH to values of 5.0 or below at the end of the fermentation process. LAB also exert a bioprotective or inhibitory effect against other microorganisms as a result of competition for nutrients, or by producing hydrogen peroxide and bacteriocins. Bacteriocins generally inhibit species taxonomically closely related to the producer strains and are differentiated in terms of their antimicrobial spectra, their mechanisms of action, and their biochemical properties (Castellano et al. 2008).

For the above specifications, LAB are the preferred bacteria for starter culture formulation. Ideally, appropriate cultures have to be selected from indigenous microorganisms, in order to be more competitive, well-adapted to a particular product, and with high metabolic capacities to beneficially affect quality and safety of the product and preserve their typicity (Leroy et al. 2006). Natural

microbial starters may diversify the market and lead to the production of regional fermented products with specific properties. Indeed research of this technological potential has been of industrial outmost interest (Aymerich et al. 2003). Traditionally, biochemical studies have focused on metabolic pathways or biosynthetic capacities of economically important species. Thus, different approaches were used to understand adaptation of bacteria to their environment and to characterize important technological and safety properties of the products. The selection of the most efficient strains contributing to a new fermented product with improved general standards has been the main objective.

### 3.1.3. Probiotic Features of LAB

Increasing scientific evidence indicates that ingestion of certain microbial cultures exerts health benefits not only in the gastrointestinal tract (GIT) but also in the respiratory and urogenital tracts (this book, Chapters 7 and 8). Conditions such as lactose intolerance, diarrhea, gastroenteritis, irritable bowel syndrome, inflammatory bowel disease, depressed immune function, cancer, and genitourinary tract infections have all been reported to benefit from probiotics (Stanton et al. 2005). The health benefits of fermented functional foods are expressed either directly through the interaction of ingested live bacteria or yeast with the host (probiotic effect) or indirectly as a result of ingestion of microbial metabolites (bioactive peptides, vitamins, cofactors, etc.) produced during the fermentation process (biogenic effect). In general LAB and bifidobacteria are considered the main groups related to probiotic effects. Specific properties considered as criteria for probiotic strain selection, such as bile tolerance, resistance to stomach acidity, and adhesion to the intestinal epithelium, in addition to antimicrobial activity, will ensure strain survival in the human GIT. Even though LAB are considered Generally Recognized as Safe organisms, specification of origin, nonpathogenicity, and antibiotic resistance of the strains should also be assessed (Stanton et al. 2005).

### 3.2. “Omics”: A New Biology Approach

Before the development of genomics, scientists focused their investigations on single or small groups of genes or proteins. The genome-sequencing projects of the late 1990s yielded entire genome sequences of many bacteria and yeasts, leading to a huge amount of genetic data. The introduction of user-friendly, browser-based bioinformatic tools to extract information from these databases constitutes a key development of the post-genomic era. It is now possible to search entire genome sequences for specific nucleic acid or protein sequences to have a global view of living organisms through *in silico* analyses (Lieber 2002). Additionally, new technologies that have arisen from genomic aim at understanding biological systems; functional genomics enclosing transcriptomics, proteomics, metabolomics, and integromics are the main new technologies developed so far. These new platforms of the so-called “-omic” technologies allow the analysis and characterization of biological systems in unknown details (thousands of genes and proteins detected simultaneously). This represents a challenge for scientific analysis and opens new perspectives for bacterial starter research purposes. Briefly, the technical basis for these “-omic” technologies are given in the succeeding discussion.

*Transcriptomics.* Often included in the term genomics, it depicts the expression level of genes. The new tool, microarrays, involves a high number of molecules (oligonucleotides) arranged on an extremely small space, commonly a slide or a chip. The interactions of RNA or DNA extracts with these biochips are investigated and allow a simultaneous analysis of pleiotropic alterations at the genome and transcriptome level. Based on the target sequences on the slide, hundreds of genes can be targeted and significant changes of their mRNA can be estimated simultaneously. Identified functional gene clusters with uniform change in expression levels allow predictions of major changes in, for example, metabolic pathways leading to the next “-omic” technology: proteomics (Ulrich-Merzenich et al. 2007).

*Metabolomics.* It is related to the qualitative and quantitative analysis of all metabolites produced by a cell, tissue, or organism under certain conditions. Thus, the metabolomic fingerprinting obtained allows the sample classification by a rapid global analysis. The techniques used are multidisciplinary and rely on chromatographic separations, often coupled with well-developed calibrations for specific analytes; “hyphenated” techniques such as high-performance liquid chromatography coupled to mass spectrometry (LC-MS) and high-performance liquid chromatography coupled to nuclear magnetic resonance (LC-NMR) are likely to have increased impact. A more detailed description of each method is given in Metabolomics-nrp (2006).

*Systems biology and integromics.* The combined information from genomics, proteomics, and metabolomics helps to obtain an integrated cell or organism understanding. These new analytic platforms are high-throughput technologies that substantially increase the dynamic range and number of metabolites and genes that can be detected (Kell 2006). In systems biology, especially metabolomic data are presently organized with the aim of creating computer models simulating biological systems, allowing, in the long term, to predict both genomic activations and metabolite flows in complex systems (Kell and Mendes 2000; Bhattacharya et al. 2003).

### 3.3. Proteomics

#### 3.3.1. Theoretical Concepts

As described earlier, all completed genome sequencing projects have provided a huge amount of DNA information, although the biologic function of the proteins codified by the detected genes remained to be revealed. Indeed the next step in the post-genomic era had to focus on the study of the function of these genes. Proteomics is one of the fields that can help to establish the connection between genome sequences and its biologic behavior.

Proteomics may be defined as the analysis of the entire protein complement expressed in a cell or any biological sample at a given time under specific

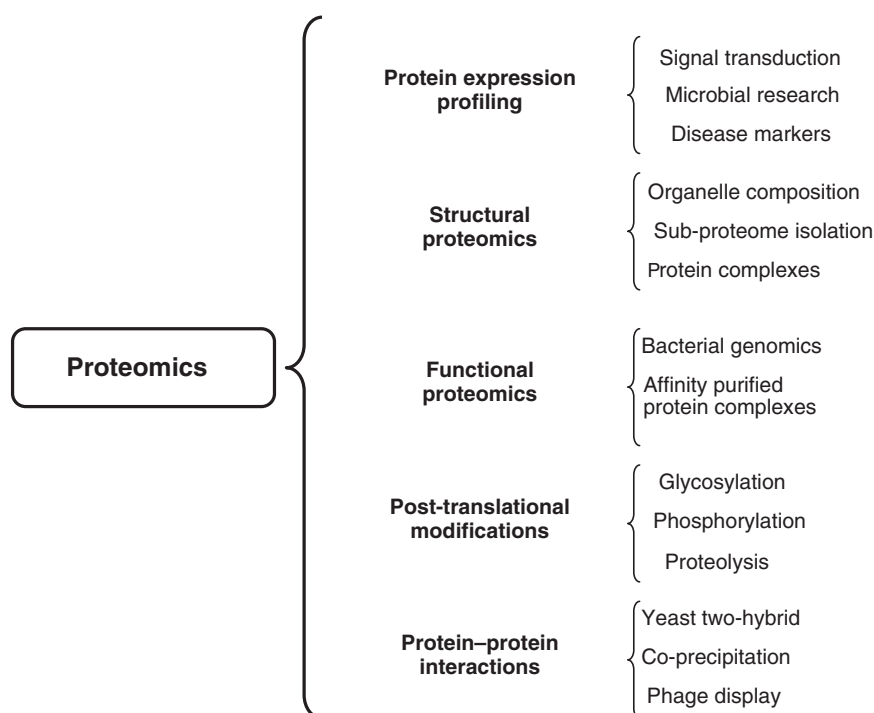
conditions (Dierick et al. 2002). Since the genome of an organism is almost completely static, while the proteome is highly dynamic, the analogy of genome and proteome is only superficial. The proteome varies as (1) not all proteins are expressed at the same time; (2) proteins are expressed in different amounts; (3) several forms of one protein occur due to posttranslational modifications; and (4) different cell types in multicellular organisms express specialized sets of proteins. The dynamics of protein expression is again highly dependent on the cellular state and the environment. Considering all the possibilities, it is likely that any given genome can potentially give rise to an infinite number of proteomes (Graves and Haystead 2002).

Many different areas of study are now grouped under proteomics; these include protein–protein interaction studies, post-translational protein modifications, protein function, and protein localization (Fig. 3.1). According to its main goal, proteomic can be divided into three types.

*Protein expression proteomic.* It comprises the quantitative analysis of protein expression between samples differing in one or more variables. This strategy compares expression of proteome or sub-proteomes between different samples. The obtained data allow the identification of new proteins involved in different cell functions such as signal transduction, specific disease makers, or proteins of interest for the metabolism of microorganisms.

*Structural proteomics.* The study of subcellular proteins and protein–protein interactions by the purification of organelles or complexes, and the identification of their components by mass spectrometry is involved.

*Functional proteomics.* It is a broad term for many specific proteomic approaches. Specific sub-proteomes can be isolated by affinity chromatography and then studied and characterized. This approach provides important information about



**Figure 3.1.** Disciplines grouped into proteomic field and its biological applications.

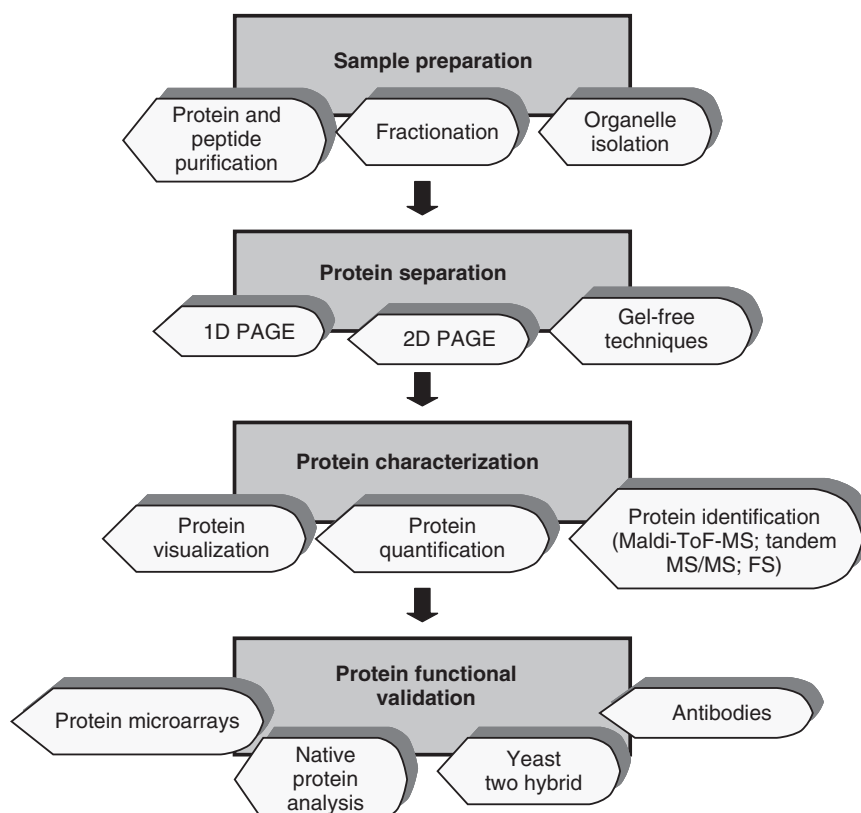
protein signaling, disease mechanisms, or protein–drug interactions.

All these ambitious goals certainly require the use of high-throughput multidimensional methods in which a large number of different disciplines such as molecular biology, biochemistry, and bioinformatics are involved.

### 3.3.2. A Technical View for the Large-Scale Analysis

Several techniques allow characterizing the proteome. A proteomics workflow analysis (Fig. 3.2) consists of two main steps: (1) sample preparation and protein separation and (2) selected protein identification. Protein separation methods include gel-based and non-gel-based approaches. In the first

case, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is used while in the latter, chromatography-based methods are applied such as liquid chromatography, capillary electrophoresis, cation exchange chromatography, and reverse-phase liquid chromatography. As an advantage of these methods, a greater number of proteins in the mixture can be evaluated, independently of the gel preparation; conversely, this approach requires a lot of time to decode the data obtained, including the analysis of uninteresting proteins also present in the peptide mixtures. One of the most promising techniques emerging as an alternative to protein electrophoresis is the isotope-coded affinity tags (ICAT); this method allows the quantitative protein profiling among different samples without the use of electrophoresis.



**Figure 3.2.** General workflow for proteomic studies.

In addition, gel-free separation techniques constitute an interesting complement for two-dimensional (2D) PAGE to have access to more cellular compartments, in particular to membrane proteins. Various mass spectrometry techniques, coupled to the above-mentioned separation methods, allow the identification of proteins (for detailed information see Graves and Haystead 2002; Graham et al. 2007 and references therein).

### 3.3.3. 2D PAGE

Until recently, the study of global protein expression was performed almost exclusively using 2D PAGE, a technique developed in the 1970s (O'Farrell 1975) with significant advances in the last decades. The strength of 2D PAGE resides in the possibility of separating up to 10,000 proteins in one gel. Every component is fractionated by isoelectric focusing on the first dimension, and then further resolved according to molecular weight in the second dimension. At this point, in the proteomic workflow a snapshot of the organism/cell may be visualized (Fig. 3.3). Proteins are excised from the gel, subjected to proteolytic digestion, and identified or sequenced; these last steps are usually carried out manually and are time-consuming, although computerized gel visualization and robotic spot-excision equipment are now available to simplify these inconveniences (Görg et al. 2004). However, it is well-established that 2D PAGE methodology has several limitations such as (1) difficulty in detecting poorly expressed proteins (Regnier and Huang 1996; Link et al. 1999); (2) limitation in isoelectric focusing; gels can only focus proteins in the pI range of 3–10, so proteins with extreme pI will not be detected (Görg et al. 2004); (3) limitation in protein size resolution since only 10–200 kDa proteins can be detected; and (4) restriction in the analysis of certain proteins due to their low solubility using the standard extraction and isoelectrofocalization protocols (e.g., membrane proteins; Graham et al. 2007).

Despite these limitations, 2D PAGE is still used as a standard tool in the analysis of microbial proteomes. This methodology allows the identification of the protein complement of the microbe under

normal conditions and then the process of subjecting the organism to a stress stimulus so that the differential expression of proteins can be visualized by either an increase or decrease in spot intensity or by the appearance/disappearance of spots on the gel.

## 3.4. State of the Art of Proteomics in LAB

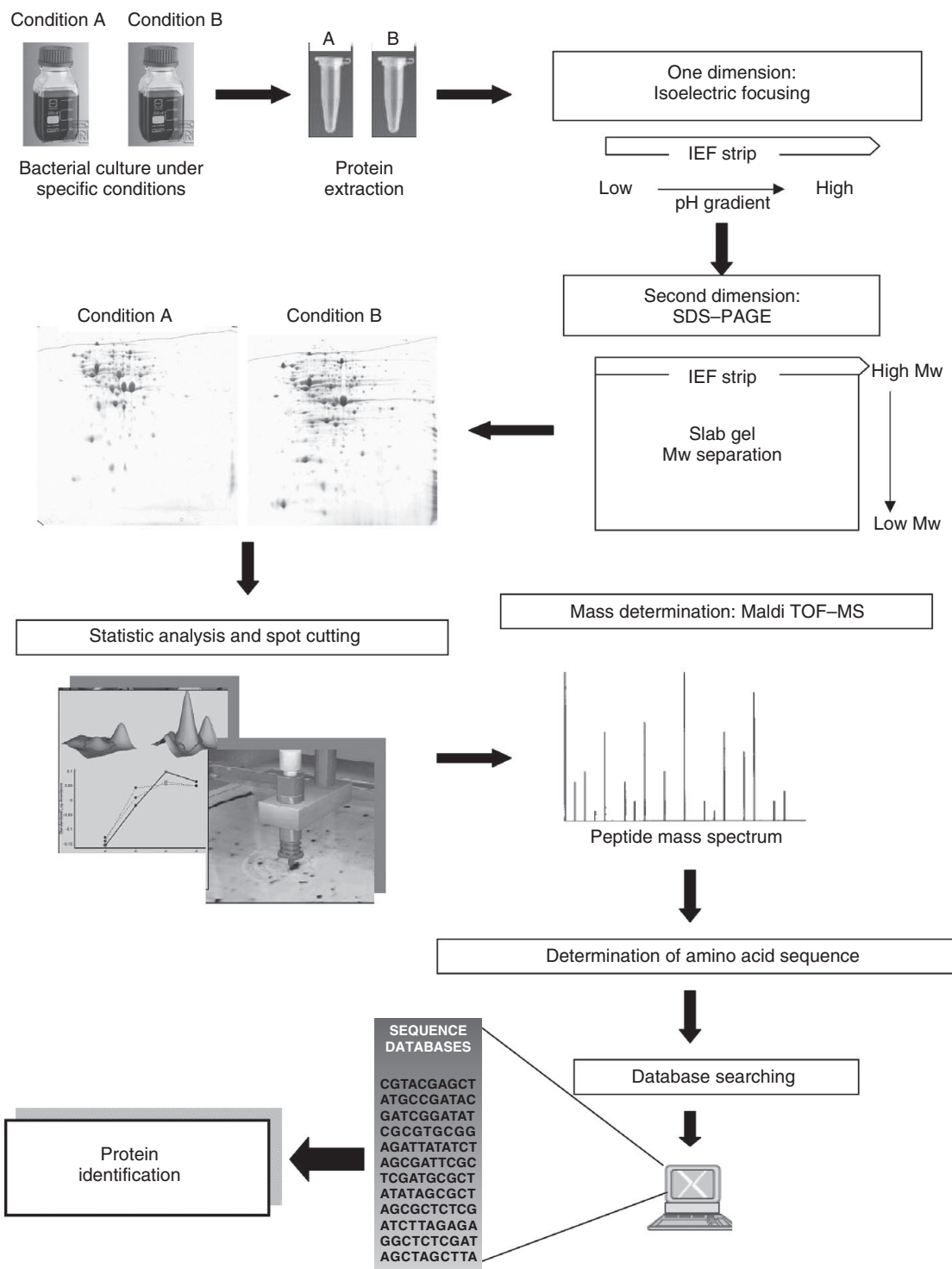
### 3.4.1. Genome Sequencing and Data Mining

The metabolic potential of LAB can be hypothesized by means of genome sequence analysis and bioinformatics as illustrated by Siezen et al. (2004). As an example, the existence of formerly unknown endopeptidases with potential to hydrolyze proline-rich caseins and bitter peptides in *Lactobacillus helveticus* DPC4571, a starter-adjunct used for Swiss cheese production, was discovered from its genome analysis (Callanan et al. 2008; Slattey et al. 2008). Also, the amino acid auxotrophy of *Lact. helveticus* predicted from the genome sequence (Callanan et al. 2008) correlated well with previous studies of amino acid requirements for this species (Hebert et al. 2000; Christiansen et al. 2008). Lastly, the complete genome sequence of *Lactobacillus sakei* 23K, a psychrotrophic LAB strain naturally present in fresh meat, determined the existence of a specialized gene catalog related to its ability to survive and compete in meat environments (Chaillou et al. 2005). Many genes seem to be responsible for overcoming the harsh condition of meat processing, such as redox and oxygen level shifts. Also, genes related to biofilm formation that allow colonization of meat surface were revealed. By using proteomics, some of the above-mentioned functions have been experimentally proven to be very important for *Lact. sakei* to meat adaptation (Marceau et al. 2002, 2004) thereby confirming the predicting value of genome data mining.

### 3.4.2. Studies of LAB Behavior in Food and GIT by Using Proteomics

The response of microorganisms to major environmental changes is well documented. As other





**Figure 3.3.** Schematic flow chart for bacterial proteome analysis by 2D PAGE.

bacteria, LAB are affected by harsh environmental conditions during passage of the GIT and food processing. Also, the food industry is interested in using stable starter cultures with good food processing adaptation capability. The knowledge of the mechanisms involved in such adaptations is thus essential for selecting the most efficient strains for a particular product. In the case of probiotic strains, these stress conditions are particularly studied in order to get a better comprehension of specific protective systems. Variations in temperature, osmotic conditions, and oxidative or acidic environments are situations to which LAB are routinely subjected and where the proteomic approach can be an invaluable tool. In the past, these proteomic approaches were rather descriptive, being focused on protein identification. More complete studies combining proteomics, genomics, and metabolomics are bringing new clues for understanding LAB physiology under these stressful environments.

**Heat shock response.** High temperature (usually between 40 and 65°C) destabilizes non-covalent interactions and leads to protein denaturation. Heat shock response is characterized by the induction of a set of heat shock proteins (HSP), some of them being lately considered rather as general stress response proteins. By a proteomic approach using 2D PAGE gels, heat shock treatment of *Lactococcus lactis* strongly induced DnaK, GroEL, and GroES as demonstrated by Whitake and Batt (1991) and Auffray et al. (1992). These proteins are well-known chaperones, which hold up folding and maturation of emerging or denatured proteins (Kaufman 1999). The expression kinetics of HSP varies during the heat shock response. Proteins such as DnaK or GroEL are induced immediately after heat shock, while other HSP are induced thereafter (Kilstrup et al. 1997).

As observed for *Lact. lactis*, a proteomic approach showed that *Lactobacillus paracasei* NFBC 338 overexpresses GroEL after a heat shock. Cloning and overexpression of the *Lact. paracasei* GroESL encoding genes in both *L. lactis* and *Lact. paracasei* was not enough to protect against heat or osmotic stresses, certainly because many other proteins were

required for such a full response. Interestingly, *L. lactis* and *Lact. paracasei* transformants overexpressing GroESL developed tolerance to solvent, notably to butanol. This cross-protection may be linked to altered membrane fluidity and modified protein embedment in membrane caused by both, solvent and heat stresses (Desmond et al. 2004). In other LAB species, a similar heat shock response including HSP induction has been reported (De Angelis et al. 2004; Savijoki et al. 2005). Furthermore, different stresses may lead to similar proteome modification, indicating that many proteins are involved in the response to different stresses (Lim et al. 2000).

In *Lactobacillus delbruekii* subsp. *bulgaricus*, a complex adaptation mechanism to heat shock response has been described by combining a proteomic approach and the study of thermoresistant mutants (Gouesbet et al. 2002). During the first 10 min of heat shock at 65°C, HSP synthesis is required to slow down cell death rate. In fact, if the synthesis of these early HSP is blocked, mortality is significantly increased. A second protective mechanism is induced after the first 10 min, when cells synthesized proteins that neutralize the deleterious effects of heat, and a recovery of viability is then observed.

**Acid stress response.** The main feature of LAB is their ability to ferment sugars leading to lactic acid production. Lactic acid has a key role during the processing of fermented products: it participates mainly as a biopreservative agent by inhibiting undesirable microorganisms and contributes to the development of the overall flavor and texture of fermented products. LAB have different adaptation strategies to counteract lactic acid inhibition effects during food processing and to face the stressful conditions of low stomach pH during their passage through the GIT. A detailed description of the current status of bacterial mechanisms to fight against acid stress has recently been reviewed (Corcoran et al. 2008).

The existence of protective mechanisms allowing an adaptive response to low pH exposure has been reported in several LAB. Some of them could also

allow a cross-protection against other stress conditions. A proteomic approach in *Lactobacillus reuteri* showed that proteins important in low pH response were also involved in bile salt protection (Wall et al. 2007; Lee et al. 2008). Low pH and bile salt are two conditions encountered during transit through GIT, thus it is not surprising that microorganisms develop coordinated response to these two stresses.

The mechanisms underlying response and adaptation to low pH on *Bifidobacterium longum* was also studied by proteomics (Sánchez et al. 2007a). The production of several enzymes involved in the different steps of glycolysis was found to be modulated by acidic pH, in a low pH-resistant mutant and in its parent strain. Some enzymes such as  $\alpha$ -1,4-glucosidase, phosphoglucomutase, and UDP-glucose 4-epimerase were up regulated. These glycolytic enzymes are related to complex carbohydrate utilization and fuel the fructose 6-phosphate pathway, the characteristic carbon catabolic pathway of bifidobacteria, also so-called *the bifid shunt* (Caescu et al. 2004). Interestingly, transaldolase was strongly underexpressed after growth at acidic pH in the wild type whereas it was overexpressed in the mutant. Theoretically, this should favor the faster formation of glyceraldehyde 3-phosphate from fructose 6-phosphate in the mutant. Accordingly, a decrease of the glucose consumption was observed for the mutant when grown at pH 4.8, together with a moderate increase of the total carbon balance of the *bifid shunt*. The intracellular pH was lowered in both strains, which correlated with the overproduction of two intracellular subunits of the  $F_1F_0$ -ATPase. A higher internal  $\text{NH}_4^+$  concentration was also observed that might compensate the lower internal pH. Thus, *B. longum* reacts to the acidic environment by changing the glycolytic flux and regulating its internal pH. Interestingly, the bile salt hydrolase was also shown to be downregulated under such acidic conditions (Sánchez et al. 2007a).

A recent study of acid adaptation on *Lact. delbrueckii* subsp. *bulgaricus* conducted through a combination of proteomics and transcriptomics (Fernandez et al. 2008) revealed induction of many chaperones (GroES, GroEL, HrcA, GrpE, DnaK, DnaJ, ClpE, ClpP, and ClpL), repression of ClpC,

induction of genes related to the biosynthesis of fatty acids (*fabH*, *accC*, *fabI*), and repression of genes involved in the mevalonate pathway of isoprenoid synthesis (*mvaC*, *mvaS*). Results obtained from this study clearly indicated that during acid adaptation, *Lact. delbrueckii* subsp. *bulgaricus* undergoes pyruvate rerouting to fatty acid biosynthesis that may affect membrane integrity.

Amine accumulation into the cells has been related to a defense mechanism to counteract acidic environments (van de Guchte et al. 2002). Amines are generated either as the result of endogenous amino acid decarboxylase activity in raw food materials or by the growth of decarboxylase-positive microorganisms (Halász et al. 1994). Biogenic amines in foods are of concern in relation to both food spoilage and food safety, as they are potential precursors of carcinogenic nitrosamines especially when nitrosable agents are present as nitrates in meat products (Scanlan 1983). Histamine and tyramine are among the most toxic amines but putrescine and cadaverine also compromise the organoleptic properties of contaminated foods.

Pessione et al. (2005) have undertaken the first proteomic analysis of amine-producing bacteria. The influence of some environmental and nutritional parameters on amine production and protein biosynthesis was analyzed in two *Lactobacillus* sp. strains, encoding histidine decarboxylase (HDC) and ornithine decarboxylase (ODC). Proteomics showed that HDC and ODC expression were dependent on the presence of high concentrations of free amino acids and on the growth phase. The stationary phase and high amounts of free amino acids also induced the biosynthesis of an oligopeptide transport protein (Opp ATP-binding cassette transporter). A lack of correlation between either decarboxylase biosynthesis or amine accumulation and external pH was observed. Indeed, amine accumulation is not induced by low pH, and production of amines does not necessarily correlate with a basic pH measured in the medium. These results do not support the hypothesis that amine production is a metabolic response of lactobacilli to medium acidification, the use of alkalization tests to determine amine accumulation being limited (Bover-Cid and Holzapfel 1999).

**Cold shock.** LAB are commonly exposed to low temperatures during starter production and storage or during various food processing steps. It is well known that low temperatures are responsible for cell viability loss, membrane damage such as morphological and fluidity changes, and effects on replication, transcription, and translation (Fonseca et al. 2001; Ouvry et al. 2002; van de Guchte et al. 2002 and references herein; Saarela et al. 2005). These changes are undesirable as they affect the technological properties of the bacteria, such as viability or acidification and consequently, the final quality of the products.

Many studies have been performed to understand cryotolerance of LAB (Panoff et al. 1998). It was reported that many bacteria developed an increased ability to survive freezing by previously applying a moderate, similar, or different stress (Palmfeldt and Hahn-Hägerdal 2000). Using a multidisciplinary approach including comparison between proteomes, Wang et al. (2005) demonstrated that a moderate cooling temperature (28°C) improved cellular cryotolerance of *Lactobacillus acidophilus*. Some proteins were differentially expressed when LAB were subjected to different cooling conditions. The optimal cryotolerance was achieved after an intermediate cooling (8 h at 26°C). In this condition the intensity of four protein spots was increased whereas the spot of one protein was reduced. One of the overexpressed proteins corresponded to an ATP-dependent Clp protease P, which is essential for survival under stressful conditions. This enzyme is involved in the proteolysis of misfolded and damaged proteins generated by a cold shock (Frees and Ingmer 1999; Ingmer et al. 1999; Skinner and Trempey 2001). A higher intensity of two spots corresponding to a pyruvate kinase and a putative glycoprotein endopeptidase, respectively, was also observed. The underregulated protein matched with a trigger factor for cell division may be related to a lower metabolic activity of the cells when they were maintained at 26°C during 8 h.

On the other hand, improvement of cryotolerance (storage at -20°C) by previous acidic shock (pH 5.25 for 30 min) was proven in *Lact. delbrueckii* subsp. *bulgaricus* (Streit et al. 2007). A further

study of its bacterial proteome after acidification revealed changes in the synthesis of 21 proteins mainly involved in energy metabolism, nucleotide and protein synthesis, and stress response. However, any of these proteins could be ascribed directly to the observed cryotolerance after acidification, although biochemical changes in the saturated and cyclic fatty acid concentration as well as lowered membrane fluidity were observed (Streit et al. 2008).

**Oxidative stress response.** LAB can also be subjected to oxidative stress during fermentation, drying, storage, or through exposure to different oxygen gradients in the gut or in food matrices. Reactive oxygen species (ROS) such as hydrogen peroxide ( $H_2O_2$ ), superoxide anion ( $O_2^-$ ), or peroxide radical ( $O^{\cdot}$ ) can react with lipids, proteins, or DNA, causing lethal damage effects (Miyoshi et al. 2003).

In bacteria, oxidative stress is sensed by specific transcriptional regulators able to activate defense mechanisms when the ROS concentration exceeds a critical level (Farr and Kogoma 1991). By means of a transcriptomic and proteomic approach, Mostertz et al. (2004) analyzed the global gene expression of *Bacillus subtilis* in response to peroxide and superoxide stress stimuli. The proteome analysis showed enhanced synthesis of approximately 55 proteins after peroxide treatment and decreased synthesis for about 150 proteins. When paraquat was used as superoxide stressing factor, more than 60 proteins were overexpressed and 200 were found at lower rates. On the other hand, 20 proteins were expressed in higher concentrations and 140 proteins in lower amounts after both stress challenges (hydrogen peroxide and paraquat). Among the overexpressed proteins, the alkyl hydroperoxide reductase, a metallorepression DNA-binding stress protein, one Class III stress-response-related ATPase, one Class III heat-shock ATP-dependent protease, one superoxide dismutase, one thioredoxin, and one thioredoxin reductase, were observed. In addition, a significant number of proteins were not synthesized after exposure to both stimuli.

An example of the study of oxidative shock by proteomic strategy in LAB is the approach reported by Arena et al. (2006) in *Streptococcus thermophilus*.

*lus*. Combining 2D PAGE plus MALDITOF and 1D PAGE plus liquid chromatography coupled to electro spray ionization and mass spectrometry (specifically  $\mu$ LC-ESI-IT-MS/MS), differential protein expression under various challenges was analyzed. This study demonstrates that  $H_2O_2$  promotes induction of general stress proteins as well as proteins specifically involved in reparation of deleterious effects of oxidative stress such as NADH oxidase, Mn superoxide dismutase, Fe-S assembly proteins SufB and SufC, glutathione reductase, Dpr peroxide resistance protein, while a downregulation of proteins related to energy metabolism was observed.

**Osmotic stress response.** The main agents causing osmotic stress in lactobacilli are salts (NaCl or KCl) often added in fermented or cured products. As for other stress responses, it has been demonstrated that salt adaptation overlaps with heat stress adaptation in *L. lactis* (Kilstrup et al. 1997). A transcriptomic study revealed that among many genes regulated by salt stress in *L. lactis*, four (*dnaK*, *busAA*, *busAB*, and *thyA*) genes were overexpressed by both heat and salt stress. In addition, gene expression of proteolytic enzymes and the  $\beta$ -glucoside-specific PTS system were repressed during osmotic stress, as in other evaluated stress stimuli (heat and acid) (Xie et al. 2004).

A proteomic study was conducted in *Lact. sakei* to identify proteins affected by addition of NaCl and low temperature (Marceau et al. 2004). Mutants were then constructed in the genes encoding proteins either over- or underexpressed. It was found that at least six proteins were involved in the adaptation of *Lact. sakei* to the environmental conditions encountered during meat processing. A mutant affected in the glycolytic enzyme phosphofructokinase showed reduced survival both at 4°C or in the presence of 4% NaCl. Similarly, the peptide methionine sulfoxide reductase (MsrA), a universal stress protein (USP family protein) and one alkaline shock protein (ASP family protein) were clearly shown to be involved in the survival of *Lact. sakei* at low temperatures.

On the other hand, a functional genomic approach derived from the draft genome sequence of *Lact.*

*acidophilus* NCFM pointed out that the response of a cell division protein (CdpA) was related to the resistance to various environmental stresses, as observed for those corresponding to S-layer and cell wall-associated proteins (Altermann et al. 2004). Furthermore, the growth of *Lactobacillus casei* ATCC 393 in 1 M NaCl revealed modifications in saturated/unsaturated fatty acid ratio and cyclopropane fatty acids membrane concentrations as reported by Machado et al. (2004). These last examples show that combination of various methods are necessary to assess the various actors of the stress response, in order to overcome proteomic limitations especially for membrane and cell wall compartment exploration.

**Bile salt resistance.** Bile salt resistance is a crucial feature for the survival of bacteria during GIT transit. The main function of bile salts, secreted into the intestine during food digestion, is the emulsification and absorption of fats. Another important feature of bile salts is their strong biocide action. Due to their detergent nature, bile salts induce lethal membrane damage, producing also DNA injury by oxidative shock. In various LAB and bifidobacteria, membrane proteins mediating bile efflux from cells were the first proteins shown to be related to bile resistance (Yokota et al. 2000; Elkins et al. 2001). The action of bile salt hydrolases, enabling deconjugation of bile salts has also been proposed as an alternative mechanism of cell detoxification in bifidobacteria (Grill et al. 2000).

A proteomic study of *B. longum* grown in the presence of bile salts showed that the expression of many enzymes involved in the *bifid shunt* was enhanced (Sánchez et al. 2005). A similar approach on *Bifidobacterium animalis* subsp. *lactis* and on a bile salt-resistant mutant showed that proteins responsible for the bile salt adaptation of this strain are mainly involved in carbohydrate metabolism. Significant differences in the levels of metabolites of the *bifid shunt* were detected, supporting proteomic data. In these two *Bifidobacterium* species, bile challenge leads thus to a modification of carbon metabolism although through different targets suggesting a different effect of bile on sugar



metabolism. Indeed, formate/oxalate metabolism was affected only in *B. animalis* subsp. *lactis*. Moreover, several proteins involved in the redox status of the cells were also affected by bile salt, this observation being correlated to intracellular pools of NADH and FAD<sup>+</sup> (Sánchez et al. 2007b).

**High-pressure stress response.** Nowadays, high hydrostatic pressure (HHP) is increasingly used as a novel food preservation method due to its nonthermal nature, its effectiveness in inhibition of undesirable microorganisms together with low effect on sensorial qualities of food products. Fruit juices, oysters, and cooked meat products are the main products subjected to this technology (Rastogi et al. 2007). HHP affects living cells causing lethal morphological, biochemical and genetic changes (Bartlett et al. 1995). The mechanisms by which HHP produces bacterial inactivation are well documented and its effectiveness depends not only on the pressure levels and duration but on the temperature, pH, osmolarity, and other factors prevailing during HHP stress. Some naturally piezotolerant microorganisms, in general inhabitants of deep-sea environments, show growth up to 94 MPa (Abe and Horikoshi 2001).

Wemekamp-Kamphuis et al. (2002) reported that pressurization of *Listeria monocytogenes* at 200 MPa led to the induction of two cold shock proteins. In addition, the preadaptation of cells at 10°C produced cross-protection, evidencing again the relation between the physiological states with pressure resistance. The recovery status of four bacterial species related to meat environment, *Lact. sakei* (two strains), *Enterococcus faecium*, *Enterococcus faecalis*, and *L. monocytogenes*, after a 400-MPa HHP treatment was species-dependent; each species induced a different set of proteins to overcome HHP shock, reflecting differences on response capabilities. Transcription factors, proteins related to protein synthesis or fate, and enzymes from energy metabolism were also induced in the four species. However, several stress proteins were specifically induced in the two *Lact. sakei* strains. Proteins from the general metabolism predominated in *Ent. faecalis* and *Ent. faecium*, and stress proteins and proteases predomi-

nated in *L. monocytogenes*. The last species showed the highest susceptibility after the HHP treatment while *Ent. faecium* demonstrated to be the less sensitive, overproducing mainly general metabolism enzymes. Finally, *Lact. sakei* presented a moderate stress response. This reveals that, after 2 h of recovery, the different species exhibited a different fitness status (Jofré et al. 2007).

### 3.4.3. Study of Flavor Production by Using Proteomics

Sapid and odor molecules, originating from raw materials and food ingredients, are also the result of the carbohydrates, proteins, and lipids catabolisms. Flavor production is strain-dependent and therefore the composition of a starter culture can greatly influence flavor characteristics of the final product (Kieronczyk et al. 2003). Food fermentations are typically carried out by mixed cultures consisting of multiple strains belonging to different species and genera. Population dynamics thus plays a crucial role in the performance of fermentations. In the past, studies on mixed-culture food fermentations have focused on population dynamics analysis, using classical and molecular methods. The availability of genome sequences as well as the technological advances in functional genomics constitutes a valuable approach for studying food microbial interactions and metabolic activities in mixed-cultures of industrial interest. In this sense, the impact of transcriptomic and proteomic approaches on the elucidation of microbial interactions was reviewed recently by Sieuwerts et al. (2008a, 2008b).

Metabolic studies can be supported by functional genomics. The ability of certain LAB to form flavor precursors from amino acids was revealed from *in silico* analyses of whole genome sequences (Liu and Siezen 2006; Liu et al. 2008; Siezen and Bachmann 2008). Moreover, *in situ* proteomic studies have been carried out on a matrix fermentation system such as milk or cheese. Yvon et al. (2008) investigated the activity of flavor-forming enzymes and the proteome and metabolome of *L. lactis* subsp. *lactis* cells extracted from a cheese model during ripening. Proteome pattern was slightly affected, the most

significant differences being found in bacterial metabolites produced by acid stress and carbon starvation after 7 days. Another proteomic approach in mixed cultures was applied to identify proteins involved in cheese ripening (Gagnaire et al. 2004). Proteins from Emmental model cheese were separated by 2D PAGE and further identified by MALDITOF or *de novo* sequencing. Among 150 spots, 21 originated from *Strep. thermophilus*, 17 from *Lact. helveticus*, and 8 from *Propionibacterium freundenrichii*. Some of these proteins were related to the organoleptic properties of cheese, that is, the peptidases PepN, PepX, and PepS from *Strep. thermophilus* and PepN, PepE, the endopeptidase PepO, and prolidase from *Lact. helveticus*. These studies showed the usefulness of proteomic profiling to identify proteins related to flavor production in fermented foods.

### 3.5. Concluding Remarks and Future Directions

It is envisioned that the information gained by the combination of “omics” approaches leads to a knowledge-based selection of LAB. This constitutes a useful tool for the improvement of established fermented foods or for the development of novel ones, based on the ability of selected starters to compete and produce metabolites of technological or health interest. In addition, the comprehension of the *in situ* behavior of starter cultures by means of the post-genomic technologies will contribute to improve fermentation processes as well as to a better understanding of microbial adaptation strategies for obtaining functional cultures with improved capabilities.

Phelps et al. (2002) proposed the identification of gene function and pathways by using bioinformatics in which data derived from transcriptomic and metabolomic analyses are combined. Teusink and Smid (2006) have also reviewed interesting modeling strategies for industrial exploitation of LAB. In this sense, Pastink et al. (2008) proposed the use of a software package (Simpheny™ Genomatica Inc., San Diego, CA) to build up *in silico* genome-scale models that, if combined with transcriptomics and

metabolomic data, could be useful to enhance the knowledge of metabolic changes occurring in mixed cultures.

In addition, the mechanisms involved in bacterial communication and interactions in food matrices still require elucidation. Microorganisms produce signaling molecules for communication purposes, secretion of proteins across biological membranes being a crucial process by which bacteria can interact together and monitor the local environment (van Pijkeren et al. 2006). This cell–cell communication phenomenon is generally recognized as “quorum sensing” (QS). Two recent reviews link QS to motility, EPS production, biofilm, and toxin production, which are all important features in food fermentation (Gonzalez and Keshavan 2006; Dunn and Stabb 2007). However, the possibility of accessing cell wall proteins and pheromone-like small peptides through proteomics needs further improvement of the analytical methods.

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## Chapter 4

# Lactic Acid Bacteria: Comparative Genomic Analyses of Transport Systems

Graciela Lorca, Lakshmi Reddy, Anphong Nguyen, Eric I. Sun, John Tseng, Ming-Ren Yen, and Milton H. Saier, Jr.

*Comparative analyses of transport proteins conducted essentially as described in an earlier study (Lorca et al. 2007) are presented here. Transporters encoded within 73 fully sequenced genomes of five groups of prokaryotes ([1] lactic acid bacteria [LAB] [11 genomes], [2] non-LAB firmicutes [11 genomes], [3] actinobacteria [5 genomes], [4] Gram [–] bacteria [33 genomes], and [5] archaea [13 genomes]) were examined. Analyses were conducted according to organism, organismal type, protein family or superfamily, functionality, and transporter energy coupling mechanism. The results revealed that in LAB, (1) 63% of all transporters of the Major Facilitator Superfamily are homolog of multidrug resistance efflux pumps and may function in this or a related capacity; (2) members of the Resistance/Nodulation/Division superfamily are virtually absent; (3) the Drug/Metabolite Transporters and Multidrug/Oligosaccharide/Polysaccharide superfamilies are underrepresented in LAB relative to other groups of prokaryotes examined; and (4) more ATP-binding Cassette (ABC) exporters but fewer ABC uptake porters are present compared with most other prokaryotic types, with frequencies of the three evolutionarily distinct ABC families of exporters being ABC1:ABC2:ABC3 = 1>>3>2 for LAB and other Gram (+) bacteria, 1>2>3 for Gram (–) bacteria, and 2>>3>1 for archaea. All four independently evolving sugar-phosphorylating families of the phosphoenolpyruvate-dependent group translocators of the bacterial*

*phosphotransferase system are present in LAB, with glucose-type systems predominating, even though fructose-type systems predominate in Gram (–) bacteria. Heavy metals are taken up primarily by members of the NRAMP and VIT families, while export of these metal ions is mediated primarily by CDF carriers. While no Ca<sup>2+</sup>:cation antiporters of the nearly ubiquitous CaCA family were detected in LAB, these organisms possess twofold more Ca<sup>2+</sup> efflux ATPases than most other bacteria. Eukaryotic P-type Na<sup>+</sup>, K<sup>+</sup>-exchange ATPases (family 1 in the Transporter Classification Database ([www.tcdb.org](http://www.tcdb.org)), H<sup>+</sup>/Mn<sup>2+</sup> efflux ATPases (family 3), and Na<sup>+</sup> or K<sup>+</sup> export ATPases (family 9) were unexpectedly present in LAB, but heavy metal and copper/silver ATPases were present in twofold lower amounts than in most other types of bacteria. No prokaryotic Kdp-type K<sup>+</sup> uptake ATPases were detected in the LAB analyzed. The results establish the existence of extensive horizontal transfer of transporter genes among closely related bacterial species with frequencies of transfer being inversely related to phylogenetic distance. They demonstrate the occurrence of unusual phylogenetic traits that must reflect the environmental conditions under which this group of organisms evolved.*

### 4.1. Introduction

Molecular phylogeny has revealed the existence of three domains of life: bacteria, archaea, and

eukaryotes (Olsen et al. 1994; Rossello-Mora 2005). The former two domains include all prokaryotic life forms with small cells and a lack of eukaryotic organelles, but prokaryotic organisms display a huge diversity of cellular activities including many metabolic capabilities that cannot be found in eukaryotes (Guerrero and Berlanga 2006). There are far more bacterial kingdoms than archaeal or eukaryotic kingdoms, probably reflecting their role as the primordial, ancestral cell type that gave rise to the other two.

One bacterial kingdom consists of the firmicutes or low G+C Gram (+) bacteria. They include spore-forming *Bacillus* and *Clostridium* species; disease-causing *Staphylococcus*, *Listeria*, and *Streptococcus* species; and a group of organisms collectively known as the lactic acid bacteria (LAB). These last mentioned organisms are uniquely of industrial importance because of their roles in the fermentation of fruits, vegetables, and meats as well as their use in the dairy industry for cheese and yogurt production (Hayes et al. 2007; Sanz et al. 2007; Reddy et al. 2008). However, these bacteria also function as producers (but also cause spoilage) of wines and beer (Coton et al. 1998; Lonvaud-Funel 1999; Rouse and van Sinderen 2008). *Lactobacillus* is one of the main bacteria used as probiotics for humans and animals because of its prophylactic or therapeutic properties (Hayes et al. 2007; Sanz et al. 2007). It provides sources of biopolymers and other chemicals (i.e., bioactive peptides, short chain acids, vitamins) that have prebiotic activity by promoting the growth of other beneficial commensal gut bacteria (i.e., *Bifidobacterium*; Hayes et al. 2007; de Vrese and Schrezenmeir 2008).

LAB all have remarkable genetically encoded synthetic capabilities, including the production of excreted bacteriocins together with immunity devices that render them insensitive to their own peptide toxins. The secretion of these antimicrobial peptides is used by LAB for biological warfare and cell communication (Eijsink et al. 2002).

The LAB group is remarkably diverse in their habitats, which are well reflected by their metabolic and transport capabilities. These organisms include the closely related *Lactobacillus casei*, *Lactobacillus*

*brevis*, and *Pediococcus pentosaceus*, as well as the more distant *Lactobacillus gasseri*, and *Lactobacillus delbrueckii*, which are often found in plants and their products as well as natural inhabitants of the human gut (Vaughan et al. 2002; Makarova et al. 2006; Lorca et al. 2007). Two other LAB, distantly related to the others, *Lactococcus lactis* subspecies *cremoris* and *Streptococcus thermophilus*, are closely related to each other; they are both found in milk and are useful for dairy product fermentations. Finally, two additional LAB, closely related to each other but more distantly related to the others, are *Leuconostoc mesenteroides*, useful for food fermentation purposes, and *Oenococcus oeni*, important in wine production (Hastings et al. 1994; Mills et al. 2005).

Certain other bacteria, including high G+C Gram (+) actinobacteria, can provide similar useful functions. *Bifidobacterium longum* is found in the intestinal tracts of animals including humans, and is an important probiotic organism that together with other bifidobacteria, dominates the intestinal tracts of infants that are breast-fed but not of those that are fed with formula (cow's milk). Bifidobacterial species are believed to be responsible in part for the greater health of breast-fed children (Parracho et al. 2007). *Brevibacterium linens* is another actinobacterium, used for cheese ripening as well as for vitamin and carotenoid production (Ratray and Fox 1999).

Transmembrane transport is essential to virtually all of the useful functions provided by LAB, and the presence of these transporters must reflect the evolutionary histories of the organisms in which they are found (Ren and Paulsen 2005). In earlier papers, some of the unusual complement of transporters encoded within LAB genomes and sequenced by the LAB genome consortium were summarized (Makarova et al. 2006; Lorca et al. 2007). In this paper, analyses of the distributions of the various transporters found in 11 LAB, their associations with the different transporter families, and their functional significance are reported. It is shown that LAB have the most unusual distributions of transporters compared with other bacterial types, including actinobacteria, Gram (–) bacteria, and archaea.

Potential evolutionary explanations are also provided.

## 4.2. The Major Facilitator Superfamily (MFS)

The largest superfamily of secondary carriers present in nature is the MFS, having the transporter classification (TC) number in the TC Database (TCDB; [www.tcdb.org](http://www.tcdb.org)) of 2.A.1 (Pao et al. 1998; Saier et al. 1999). It currently consists of 67 families that can transport virtually any type of small substrate of biological importance. Members can be solute importers, catalyzing the active uptake of solutes against concentration gradients using a proton symport mechanism with the proton motive force (pmf) serving as the energy source, but they can also catalyze solute efflux against a concentration gradient using a proton antiport mechanism, thus extruding solutes out of the cell. Finally, a few MFS carriers are solute equilibrators, catalyzing facilitated diffusion via a solute uniport mechanism, and several, both symporters and uniporters, can also catalyze solute:solute exchange; the type of vectorial reaction catalyzed is determined by the carrier.

Each of the 61 families of the 67 currently recognized MFS families appears to consist of members that catalyze either uptake or efflux, but not both. However, there are six potential exceptions (SP, DHA1, DHA2, OPA, NNP, and OCT), based on reports from different laboratories (see TCDB). The sugar porter (SP) family (TC# 2.A.1.1) includes members that usually utilize sugar:H<sup>+</sup> symport but can also use equilibrative sugar uniport. Uniporters of the SP family occur primarily in animals that maintain constant high sugar levels in the extracellular plasma, due to strict homeostatic mechanisms. However, they can also be found in yeasts, and occasionally, in bacteria that grow in environments containing high sugar concentrations. Some SP carriers have evolved receptor functions, thereby gaining regulatory sensor activities (Kruckeberg et al. 1998). However, no member of this huge family within the MFS has been shown to catalyze substrate:H<sup>+</sup> antiport.

Other reported examples of facilitated diffusion include a fructose uniporter in the yeast, *Zygosaccharomyces* (2.A.1.2.23), which is a member of the DHA1 family of drug:H<sup>+</sup> antiporters, and the putative bacterial bile acid uptake porter (2.A.1.3.13) in the DHA2 family of drug exporters. The SP and DHA1 families are among the largest families in the MFS. It seems that in the MFS and certain other families, the loss of cation symport activity to generate substrate uniporters has occurred relatively frequently during evolution, that symporters and uniporters frequently can catalyze substrate:substrate exchange, but that interconversion of antiporters and symporters has been relatively rare during evolutionary history (see examples in TCDB).

In the Organophosphate:Phosphate Antiporter (OPA) family (2.A.1.4), most members preferentially catalyze substrate:substrate antiport, but they can also catalyze substrate:H<sup>+</sup> symport, and some seem to *preferentially* use a symport rather than an antiport mechanism. Also, in the Nitrate/Nitrite Porter (NNP) family (2.A.1.8), members of similar sequence can catalyze either uptake, efflux, or NO<sub>3</sub>:NO<sub>2</sub><sup>-</sup> exchange. Finally, members of the Organocation Transporter (OCT) family in animals (2.A.1.19) appear to similarly be mechanistically promiscuous, catalyzing substrate uptake, export, exchange, and/or uniport. Sometimes the mechanism and substrate:H<sup>+</sup> stoichiometry depends on the conditions and the specific substrate being transported. A few MFS carriers can use Na<sup>+</sup> instead of H<sup>+</sup> as the cotransported cation.

MFS carriers are usually about 400 amino acid residues (aas) long and have 12 transmembrane  $\alpha$ -helical spanners (TMSs) with two homologous repeat units, each of 6 TMSs. They are found ubiquitously in bacteria, archaea, and eukaryotes. Over 20,000 sequenced MFS members are available for sequence analysis, and x-ray structures have been reported for three of them (Law et al. 2008). Some evidence suggests that the basic 6 TMS repeat unit arose by duplication of a primordial 3 TMS encoding genetic element and that MFS carriers arose from simple 2 TMS ion channels (Hvorup and Saier 2002).

**Table 4.1.** Functional types of MFS carriers and their relative occurrences in five different prokaryotic groups of organisms.

Group	LAB	Non-LAB firmicutes	Actino-bacteria	Gram (–) bacteria	Archaea
Drugs	63%	62%	48%	41%	59%
Anions	11%	16%	36%	32%	19%
Sugars	8%	8%	8%	9%	10%
Amino acids	0%	0%	0%	2%	0%
Peptides	10%	8%	5%	11%	9%
Nucleosides	9%	7%	3%	5%	3%
<b># Systems</b>	<b>291</b>	<b>298</b>	<b>138</b>	<b>990</b>	<b>142</b>
<b># Organisms</b>	<b>11</b>	<b>13</b>	<b>5</b>	<b>41</b>	<b>16</b>
<b># Systems/organism</b>	<b>26</b>	<b>23</b>	<b>28</b>	<b>24</b>	<b>9</b>

**Table 4.2.** Distribution of RND superfamily members in five groups of prokaryotes.

Family group	% Representation					# Systems/organism (ratio)
	HME	HAE1	SecDF	HAE2	HAE3	
LAB	0	0	0	100	0	1/11 (0.1)
Non-LAB firmicutes	0	28	44	28	0	18/13 (1.4)
Actinobacteria	0	0	25	75	0	32/5 (6.4)
Gram (–) bacteria	12	58	21	0	8	264/41 (6.4)
Archaea	0	0	59	0	41	34/16 (2.1)

As presented in Table 4.1, LAB and non-LAB firmicutes have disproportionately high representation of MFS multidrug efflux pumps (62%–63%), while the organic and inorganic anion uptake porters occur in low number (11% for LAB and 16% for the other firmicutes) compared with other bacterial groups (actinobacteria and Gram [–] bacteria with 36% and 32% representation, respectively). The high percentages of drug exporters in LAB reflect the apparent obsession of these organisms with biological warfare, while the low numbers of anion uptake systems may reflect their fermentative (sugar) modes of metabolism rather than respiratory (organic acid) modes of metabolism. LAB also have two- to threefold more nucleoside/nucleobase transporters than most other prokaryotes examined (Table 4.1).

### 4.3. Other Large Superfamilies of Secondary Carriers

Excluding the MFS, the three largest superfamilies of secondary transporters include the Drug/

Metabolite Transporters (DMT; both uptake and efflux systems; Jack et al. 2001), the Resistance/Nodulation/Division (RND) exporters (exclusively export carriers; Tseng et al. 1999), and the Multidrug/Oligosaccharide/Polysaccharide (MOP) porters (exclusively efflux pumps; Hvorup et al. 2003a). These three superfamilies will be examined sequentially in this section.

The RND superfamily is essentially absent in the LAB characterized in this study, with only one exception: a single lipid-exporting HAE2 family member (Table 4.2; Lorca et al. 2007). The heavy metal exporters (HME) are found only in Gram (–) bacteria. HAE1 MDR pumps are present only in Gram (–) bacteria and non-LAB firmicutes with lower representation in the latter compared with the former. HAE3 homologs are present only in archaea and Gram (–) bacteria but not in the Gram (+) bacteria surveyed. Finally, the general protein secretory (Sec) auxiliary proteins, SecDF, which together comprise the equivalent of a full-length RND pump that is thought to facilitate integral cytoplasmic membrane protein insertion (Xie et al. 2006), are

**Table 4.3.** Distribution of DMT superfamily members in five groups of prokaryotes.

TC family #:	1	3	5	7	21	23	24	
TC family name:	SMR	DME	GRP	RarD	BAT2	Trp-E	TPPT	# Proteins/organism
Group								
LAB	13	33	29	4	10	4	8	52/11 (4.7)
Non-LAB firmicutes	21	33	13	5	7	6	15	85/11 (7.7)
Actinobacteria	17	50	0	25	8	0	0	12/5 (2.4)
Gram (–) bacteria	16	60	0	6	5	6	8	283/33 (8.6)
Archaea	9	64	0	0	0	7	20	44/14 (3.1)

**Table 4.4.** Distribution of MOP superfamily members in five groups of prokaryotes.

	1	2	4	7	12	
Family/group	MATE	PST	MVF	U-MOP1	U-MOP4	# Proteins/organism
LAB	34	47	2	15	0	47/11 (4.3)
Non-LAB firmicutes	53	33	4	8	1	76/11 (6.9)
Actinobacteria	50	8	42	0	0	12/5 (2.4)
Gram (–) bacteria	41	28	21	7	3	155/32 (4.8)
Archaea	43	41	1	15	0	87/14 (6.2)

present in all prokaryotic types examined except the LAB. In *Escherichia coli*, loss of these proteins decreases protein secretion efficiency and gives rise to a temperature-sensitive growth phenotype (Xie et al. 2006). It is surprising that SecDF homologs are lacking in LAB that can grow at rapid rates. A compensatory mechanism must exist in LAB allowing the cells to grow rapidly in the absence of these proteins.

The DMT superfamily (Jack et al. 2001) shows decreased proportions of members of (1) the small cationic multidrug resistance (SMR) efflux pumps, and (2) the Drug/Metabolite Exporter (DME) family in LAB and other firmicutes relative to other bacterial types. DME porters characterized so far are mostly metabolite exporters. Archaea show the lowest percentages of SMR family members but the largest percentages of DME, Trp-E, and TPPT family members of all prokaryotic groups examined (Table 4.3). The RarD and BAT2 families were not found in archaea. Glucose/Ribose Porter family members, which take up sugars, are present only in firmicutes with LAB showing more than twice as many family members as non-LAB firmicutes (see Table 4.3).

In LAB, the MOP superfamily (Table 4.4; Hvorup et al. 2003a) shows low percentages of multi antimicrobial extrusion (MATE) family multidrug resistance (MDR) pumps and Mouse Virulence Factor family porters (of unknown function) compared with other bacteria examined. U-MOP4 family members (of unknown function) are poorly represented in all groups of prokaryotes. However, LAB exhibit increased proportions of polysaccharide exporters of the polysaccharide transport (PST) family, and of U-MOP1 family members relative to other types of bacteria (Table 4.4). It should be noted that in all of the superfamilies mentioned, several families of unknown specificity are present. Some of these are represented in LAB while others are not. This fact may be understood when the functions of these transporters are revealed. The underrepresentation of both DMT and MOP superfamilies relative to other firmicutes is also worthy of note.

#### 4.4. ATP-Binding Cassette (ABC) Transporters

Members of the ABC Superfamily (Davidson and Maloney 2007) belong to 77 currently described families, including 27 families of uptake systems

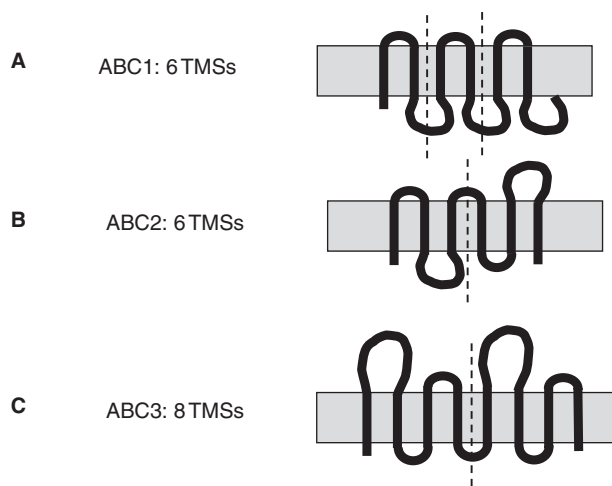


and 50 families of exporters (see TCDB). Each family is, in general, specific for a different type of substrates, although exceptions to this generalization are known. They can be specific for all kinds of small molecules, and members of several families function in the export of macromolecules (proteins, lipids, and complex carbohydrates). They share the characteristic of being driven by ABC protein-dependent ATP hydrolysis (Davidson and Maloney 2007).

ABC primary active transporters form a unified group of proteins only in the sense that they use homologous energy-coupling ABC proteins. These are superimposed on the transmembrane porters that derive from at least three distinct families of independently evolving transporters. One of these families (ABC1) exhibits 6 TMSs per polypeptide chain, having arisen from a primordial 2 TMS hairpin peptide as a result of two intragenic triplication events ( $2 \times 3 = 6$ ; Fig. 4.1A). The second family (ABC2) arose from a 3 TMS precursor by a single intragenic duplication event ( $3 \times 2 = 6$ ), giving rise

to a set of independently evolving 6 TMS proteins showing no sequence similarity with ABC1 proteins (Fig. 4.1B). A third family (ABC3) has members with 4, 8, or 10 TMSs per polypeptide chain (Fig. 4.1C). ABC3 proteins have a basic 4 TMS topology, but in the 8 and 10 TMS homologs, this unit has been duplicated, and in the 10 TMS proteins, duplication resulted in the creation of two extra nonhomologous TMSs separating the two repeat units (Fig. 4.1C). These three types of systems are all present in the three domains of life, but in varying numbers, depending on the organismal type.

Over 20,000 sequenced members of the ABC superfamily are collectively available for analysis. They occur in relative numbers of  $1>2>3$  when all living organisms are considered, but the distributions of these porter types are organismal-specific. Thus, LAB and actinobacteria display relative proportions of  $1>>3>2$ , archaea have  $2>>3 = 1$ , and Gram (–) bacteria and eukaryotes exhibit proportions of  $1>2>3$  (Table 4.5). Surprisingly, the distributions of the three topological types occurring in



**Figure 4.1.** Schematic depiction of the structures of the three families of integral membrane ABC exporters that catalyze substrate efflux using ATP hydrolysis for energy coupling. A. ABC1 proteins are 6 TMS permeases that arose by intragenic triplication of a 2 TMS hairpin-encoding gene; B. ABC2 proteins are 6 TMS permeases that arose by intragenic duplication of a gene encoding a 3 TMS primordial structure giving a 6 TMS protein with the two repeat units having opposite orientation in the membrane; C. ABC3 proteins are 4, 8, and 10 TMS permeases where duplication of the primordial 4 TMS element gave rise to the 8 and 10 TMS proteins. In the 10 TMS proteins, the extra two TMSs separate the two 4 TMS repeat units.

**Table 4.5.** Group distributions of ABC1, ABC2, and ABC3 types of ABC exporters.

Type/group	ABC1 (%)	ABC2 (%)	ABC3 (%)	Proportions	# Proteins/organism
LAB	56	19	25	1>>3>2	305/11 (28)
Non-LAB firmicutes	43	29	28	1>2=3	335/11 (31)
Actinobacteria	55	17	28	1>>3>2	97/5 (19)
Gram (–) bacteria	37	34	29	1>2>3	641/33 (19)
Archaea	23	52	25	2>>3=1	200/14 (14)
<b>Total</b>	<b>41</b>	<b>32</b>	<b>27</b>	<b>1&gt;2&gt;3</b>	<b>1578/73 (22)</b>

**Table 4.6.** Functional types of ABC exporter expressed in percent, identified in different types of prokaryotes.

Substrate type/Organismal type	Drugs	CHOs	Lipids	Proteins	Peptides	AAs	Unk	Total #
LAB	<b>64</b>	<b>4</b>	<b>1</b>	<b>10</b>	<b>12</b>	<b>3</b>	<b>6</b>	<b>305</b>
	1>>2>3	2>3	3	1=3	1	1	3>>2,1	1>>3>2
Non-LAB firmicutes	<b>70</b>	<b>5</b>	<b>1</b>	<b>10</b>	<b>4</b>	<b>3</b>	<b>7</b>	<b>335</b>
	1>>2,3	2>>3	2	1=3>2	2>3>1	1	3>2>1	1>2=3
Actinobacteria	<b>42</b>	<b>6</b>	<b>1</b>	<b>10</b>	<b>14</b>	<b>8</b>	<b>19</b>	<b>97</b>
	1>2>3	3>>2	1	3	1	1	3>1	1>>3>2
Gram (–) bacteria	<b>39</b>	<b>10</b>	<b>10</b>	<b>16</b>	<b>7</b>	<b>4</b>	<b>14</b>	<b>641</b>
	2>1>3	2>>3>1	3>1>2	1=3	3>2>1	1	1=3>2	1>2>3
Archaea	<b>51</b>	<b>8</b>	<b>3</b>	<b>8</b>	<b>9</b>	<b>5</b>	<b>17</b>	<b>200</b>
	3>2>1	2	1>2	3>2>>1	1>2	2>>1	3>2>>1	2>>3=1
Total	<b>52</b>	<b>7</b>	<b>5</b>	<b>12</b>	<b>8</b>	<b>4</b>	<b>12</b>	<b>1578</b>
	1>2>3	2>>3>1	3>2>>1	3>1>>2	1>2>3	1>>2	3>2>1	1>2>3

the five organismal groups examined are distinctive, with only a few exceptions. Thus, Gram (–) bacteria and archaea have fewer ABC1 and more ABC2 porters than the three types of Gram (+) bacteria. Archaea are most unusual as ABC2 porters predominate over ABC1 porters.

With respect to the relative proportions of the major functional types of ABC exporters, MDR pumps predominate in all groups of living organisms (Table 4.6). In LAB and other firmicutes, 64%–70% of all ABC exporters probably serve protective functions of drug/hydrophobic compound efflux. LAB have three times as many ABC-type peptide exporters as do other firmicutes. However, these organisms have fewer ABC amino acid, lipid, and complex carbohydrate exporters than other prokaryotic phyla (Table 4.6). This fact correlates with the use of signaling peptides in Gram (+) bacteria to a much greater extent than in Gram (–) bacteria.

ABC uptake permeases often exhibit 5 TMSs per polypeptide chain, although some have been inter-

nally duplicated to give 10 TMSs. The probability that the basic 5 TMS unit also evolved by intragenic duplication was recently suggested; the statistical analyses reveal that TMSs 1–2 are homologous to TMSs 4–5 (E.I. Sun, B. Wang, W.H. Zheng, D.C. Yee, and M.H. Saier, unpublished results). Two possible routes can be considered. First, a 2 TMS hairpin polypeptide-chain might have duplicated so that a 5 TMS protein resulted in the N-terminal hairpin facing inward and the C-terminal hairpin facing outward. In this case, the central TMS (TMS3) would have been generated *de novo* during the duplication event. Second, a 3 TMS precursor could have duplicated to give a 6 TMS protein, and then the first TMS would have been lost as a result of a small N-terminal deletion mutation, early during the evolution of these ABC porters. Due to these uptake porters showing significant sequence similarity with ABC2 exporters, the second interpretation is favored (E.I. Sun, W.H. Zheng, and M.H. Saier, unpublished observations).

Some of these uptake porters are capable of functioning either as low-affinity, low-efficiency, secondary carriers (when the ABC protein is absent) or as high-affinity, low-efficiency primary active transporters (when the ABC protein is superimposed on the transmembrane porter). This has been demonstrated for the *E. coli* biotin uptake system, BioY (Hebbeln et al. 2007). However, genomic evidence suggests that other homologous transport proteins (prokaryotic ThiW, TrpP, and phosphate:H<sup>+</sup> or Na<sup>+</sup> symporters of the PNaS family; see TCDB) may also be capable of catalyzing primary active uptake with appropriate ATP-hydrolyzing subunits (E.I. Sun and M.H. Saier, unpublished observations). Moreover, recently published data suggests that this family of porters is much larger than previously thought and that many are specific for vitamins or vitamin precursors (Rodionov et al. 2009). These suggestions need to be substantiated by wet lab experimentation.

#### 4.5. Heavy Metal Transporters

Heavy metal ions are both essential for life and toxic at high concentrations. Eight families of secondary active transporters are known to mediate divalent cation uptake and efflux (Nramp, VIT, ZIP, and NiCoT for uptake; and CaCA, CDF, CadD, and RND/HME for efflux). In LAB, two of the four families of heavy metal uptake systems (Nramp and

VIT) are represented in much larger numbers (2–4 greater representation) than the other two families (ZIP and NiCoT; Table 4.7). This situation is similar in non-LAB firmicutes except that, most surprisingly, VIT family members are reduced by over tenfold. Actinobacteria and Gram (–) bacteria exhibit more equal numbers of the four family members, but in archaea, the ZIP family is dominant over the Nramp and VIT families, which are in turn better represented than the NiCoT family (Table 4.7).

Of the heavy metal efflux systems, the CDF family predominates in all prokaryotic phyla examined, but the CadD family is well represented in Gram (+) bacteria (Table 4.7). CadD family members are not present in Gram (–) bacteria. On the other hand, within the RND superfamily, HME family is well represented in Gram (–) but not in Gram (+) bacteria. Neither family is represented in archaea. Thus, the CadD and HME families appear to occur in restricted prokaryotic phyla. Only one, not both, is present in any one type of organism (Table 4.7).

The Ca<sup>2+</sup>:H<sup>+</sup> or Na<sup>+</sup> cation antiporters (CaCA family; Lytton 2007) are similarly represented in actinobacteria, Gram (–) bacteria, and archaea, but non-LAB firmicutes have substantially reduced numbers, and LAB lack these carriers altogether. These porters function primarily to exclude cytoplasmic Ca<sup>2+</sup>, but some of them can also export

**Table 4.7.** Secondary carriers specific for divalent cations. All values are expressed as the average number of the transporters in each family divided by the number of genomes examined.

Organismal type/family	# Transporter 5/# organisms				
	LAB	Non-LAB (firmicutes)	Actino bacteria	Gram (–) bacteria	Archaea
Nramp (Mn <sup>2+</sup> , Fe <sup>2+</sup> , Zn <sup>2+</sup> , Cu <sup>2+</sup> , Cd <sup>2+</sup> , Ni <sup>2+</sup> , Co <sup>2+</sup> uptake)	1.7	1.2	1	0.4	0.4
VIT (Fe <sup>2+</sup> , Mn <sup>2+</sup> uptake)	1.3	0.1	0.5	0.2	0.4
ZIP (Zn <sup>2+</sup> , Fe <sup>2+</sup> , Co <sup>2+</sup> , Mn <sup>2+</sup> , Ni <sup>2+</sup> uptake)	0.1	0.3	0.5	0.5	0.9
NiCoT (Ni <sup>2+</sup> , Co <sup>2+</sup> uptake or efflux)	0.2	0.1	0.2	0.6	0.2
CaCA (Ca <sup>2+</sup> , Mn <sup>2+</sup> , Zn <sup>2+</sup> , Mg <sup>2+</sup> efflux)	0	0.4	0.8	0.8	0.8
CDF (Co <sup>2+</sup> , Ni <sup>2+</sup> , Cd <sup>2+</sup> , Zn <sup>2+</sup> , Cu <sup>2+</sup> efflux)	1.5	2	0.8	1.3	1.8
CadD (Cd <sup>2+</sup> efflux)	0.4	0.2	0.4	0	0
RND/HME (Ni <sup>2+</sup> , Co <sup>2+</sup> , Zn <sup>2+</sup> , Ag <sup>+</sup> , Pb <sup>2+</sup> , Hg <sup>2+</sup> efflux)	0	0	0	0.6	0

other divalent ions. In the next section we shall see that the representation of P-type  $\text{Ca}^{2+}$ -ATPases is inversely proportional to the numbers of CaCA family members in the different bacterial types. Thus, it seems that  $\text{Ca}^{2+}$  export is essential, but that either CaCA family members or  $\text{Ca}^{2+}$ -ATPases can perform this function. Whether one or the other is used must depend on environmental or physiological conditions. For example, when the primary source of energy is ATP, resulting from fermentative metabolism, ATP-dependent primary active transporters tend to predominate over pmf-driven secondary carriers. However, when respiration provides a pmf as the primary energy source, and ATP is secondarily derived from the pmf via an F-type ATPase, secondary carriers predominate (Paulsen et al. 1998, 2000).

#### 4.6. P-type ATPases in Prokaryotes

P-type ATPases control cation homeostasis in many organismal types (Fagan and Saier 1994; Palmgren and Axelsen 1998; De Hertogh et al. 2004; Kühlbrandt 2004; Haupt et al. 2005). As noted above, firmicutes, which have low numbers of CaCA family members, have two to three times as many  $\text{Ca}^{2+}$ -ATPases as are found in actinobacteria and Gram (–) bacteria (Table 4.7). By contrast, archaea, which have a good representation of CaCA carriers, also have excellent representation of the  $\text{Ca}^{2+}$ -ATPases.

Of great interest is the fact that LAB encode P-type ATPases in their genomes that normally occur in eukaryotes but not in most other bacteria. These include members of the families of  $\text{Na}^+$ ,  $\text{K}^+$ -exchange ATPases of animals (family 1 in TCDB),  $\text{H}^+/\text{Mn}^{2+}$ -ATPases of plants and fungi (family 3), and  $\text{Na}^+$  or  $\text{K}^+$  extruding ATPases of fungi and unicellular eukaryotes (family 9).  $\text{Na}^+$ ,  $\text{K}^+$ -type ATPases are found in LAB and archaea;  $\text{H}^+/\text{Mn}^{2+}$ -ATPase are present in LAB and, in lower numbers, in Gram (–) bacteria and archaea; and  $\text{Na}^+$  or  $\text{K}^+$  exporters are present only in LAB (Table 4.7). The presence of eukaryotic  $\text{K}^+$ -ATPase types correlates with the absence of prokaryotic Kdp-type ATPases (Bramkamp et al. 2007) in LAB. These observations

may reflect the close associations of various LAB with animals, plants, and fungi (Gobbetti et al. 1994; Vaughan et al. 2002; Reddy et al. 2008) although the possibility of vertical descent should not be excluded. It is also worth noting that copper and heavy metal exporters are present in firmicutes (especially LAB) in lower proportions compared with all other prokaryotic types examined (Table 4.7). Interestingly, three copper ATPase genes that mediate copper homeostasis in *Lactobacillus bulgaricus* were induced upon acidification of the culture medium (Penaud et al. 2006).

Our studies have identified P-type ATPases that do not fall into one of the nine functionally characterized families of these enzyme/transporters. Altogether, we have identified about two-dozen families, each represented only in eukaryotes or in prokaryotes but not in both. Some of the largest of these families, well represented in prokaryotes, are presented in Table 4.8. Molecular genetic, biochemical, and physiological experimentation will be required to determine the transport substrates of these enzymes.

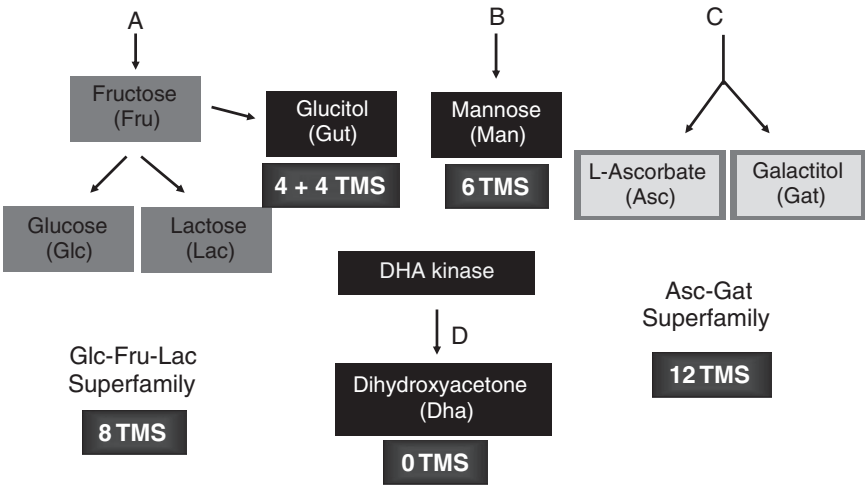
#### 4.7. The Bacterial-Specific Phosphotransferase System (PTS)

The PTS catalyzes group translocation, the coupling of sugar transport to sugar phosphorylation. Phosphoenolpyruvate is the initial phosphoryl donor. It phosphorylates Enzyme I, and then the phosphoryl group is sequentially passed to HPr, Enzyme IIA, Enzyme IIB, and finally to an incoming sugar in a reaction dependent on the transmembrane porter, Enzyme IIC. The cytoplasmic, general energy coupling proteins are Enzymes I and HPr, while the Enzyme II complexes consist of IIA, IIB, and IIC subunits, sometimes fused to each other. In the case of the mannose systems, there are also IID subunits.

As discussed previously (Hvorup et al. 2003b; Barabote and Saier 2005; Saier et al. 2005), there exist four independently evolving families of PTS group translocators (Fig. 4.2). These permeases consist of IIA, IIB, IIC, and in the Man family, IID protein domains. Family 1, the Glucose/Fructose/

**Table 4.8.** P-type ATPase superfamily representation (% of total systems for each organismal type).

Family/group	1 Na, K <sup>+</sup> (Out/In)	2 Ca <sup>2+</sup> (Out)	3 H <sup>+</sup> , Mn <sup>2+</sup> (Out)	4 Mg <sup>2+</sup> (In)	5 Cu <sup>+</sup> , Ag <sup>+</sup> or Cu <sup>2+</sup> (Out/In)	6 HM (Out)	7 K <sup>+</sup> (In)	9 Na <sup>+</sup> ; K <sup>+</sup> (efflux)	23 Unk	25 Unk	32 Unk	# proteins/ organism
LAB	6	31	6	7	18	13	0	2	9	9	0	89/11 (8.1)
Non-LAB firmicutes	0	32	0	9	23	17	10	0	5	2	2	57/11 (5.2)
Actinobacteria	0	11	0	0	33	24	3	0	5	5	5	37/5 (7.4)
Gram (–) proteobacteria	0	11	2	9	32	15	12	0	0	2	2	121/33 (3.7)
Archaea	5	35	3	2	31	19	0	0	0	2	3	58/14 (4.1)
Average %	2	24	3	6	27	17	6	0.3	3	4	2	362/73 (5.0)



**Figure 4.2.** Four independently evolving families within the PTS functional superfamily. This schematic figure illustrates independent evolutionary origins for the four currently recognized families. A. The Glc/Fru superfamily; B. the Man family; C. the Asc/Gat superfamily; and D. the Dha family.

Lactose (Glc/Fru/Lac) superfamily, consists of transporters that fall into seven phylogenetic clusters, each specific for a particular sugar or sugar type as follows: (1) glucose, (2) glucosides, (3) fructose, (4) mannitol, (5) lactose, (6) diacetylchitobiose, and (7) glucitol (Nguyen et al. 2006). All of these systems except the glucitol-type systems probably have 8 TMSs per polypeptide chain (Nguyen et al. 2006). In the glucitol systems, the 8 TMSs are split between two proteins, each with 4 TMSs. Glucitol porters exhibit sufficient sequence similarity to establish that they are homologous to other members of the Glc/Fru/Lac family (Fig. 4.2A), although

these porters comprise the most sequence divergent group of proteins in this superfamily (Nguyen et al. 2006).

The mannose (Man) family, family 2, is the only family to have a IID constituent (Saier et al. 2005). Only this family includes systems that each can exhibit broad specificity for hexoses (glucose, mannose, fructose, glucosamine, N-acetyl glucosamine, etc.). These 6 TMS proteins evolved independently of the 8 TMS permeases of the Glc/Fru/Lac family (Fig. 4.2B). All constituents of the Man family permeases (IIA, IIB, IIC, and IID) are unique to this family, although the IIA protein has been



incorporated into the late-evolving Dha PTS (see below).

Two distantly related families, the ascorbate (Asc) group translocators and the galactitol (Gat)-specific systems (Fig. 4.2C), have 12 TMSs per polypeptide chain and together comprise family 3 (Hvorup et al. 2003b). They are related by common descent and have a basic 6 TMS repeat unit, duplicated to give the 12 TMS topology. They may have evolved from secondary carriers (Fig. 4.2C). Surprisingly, it appears that members of the galactitol family, but not of the ascorbate family, may still be able to function as secondary carriers (Saier et al. 2005).

Finally, the dihydroxyacetone (DHA) PTS systems (the DhA systems; Fig. 4.2D) are not transporters at all. They phosphorylate DHA in the cytoplasm using phosphoenolpyruvate as the phosphoryl donor (Garcia-Alles et al. 2004). However, unlike all PTS permeases, the protein complex exhibiting 0 TMSs is a multisubunit cytoplasmic enzyme complex, and arose from soluble ATP-dependent DHA kinases. These unique kinases are found in bacteria including some that also have the DHA-PTS (Barabote and Saier 2005).

Dha systems of the PTS have three protein constituents, DhaM (IIA), DhaL (IIB), and DhaK (IIC; Siebold et al. 2003). DhaL corresponds in sequence to the N-termini of DHA kinases while DhaK corresponds to the C-termini of these kinases. DhaM contains a domain that is distantly related to IIA proteins of the mannose (Man) systems (family 2, Fig. 4.2B) and can be fused to other PTS domains. Like the GAT systems, these systems are not full-fledged PTS systems because (1) DhaL contains tightly bound ADP, which is phosphorylated rather than a histidyl or cysteal residue in the proteins, and (2) DhaK binds DHA covalently via a histidyl residue to provide specificity (Siebold et al. 2003; Garcia-Alles et al. 2004). Covalent bond formation between enzymes and the sugar substrate is not observed for any other PTS enzyme.

Numbers of PTS proteins encoded within the genome of an organism do not correlate with genome size (Barabote and Saier 2005). For example, both large genome organisms such as most actinobacteria

and small genome organisms such as *Mycoplasma* species usually have few PTS permeases. Firmicutes, including LAB, have the most PTS permeases in spite of their reduced genome sizes. Yet not all LAB and other firmicutes have large numbers of PTS porters; these organisms differ in over a 20-fold range with respect to their PTS permease representation. Those firmicutes with highest representation have over 3% of their genomes encoding PTS proteins, up to about two-dozen PTS porters (Barabote and Saier 2005).

Strain differences within a single species show surprising differences in PTS content. For example, various strains of *Streptococcus pyogenes* may have or lack a DHA-PTS, while various strains of *Streptococcus agalactiae* differ with respect to the presence or absence of an extra lactose (Lac) permease. Finally, *Streptococcus pneumoniae* strains differ with respect to the presence of a fructose (Fru) system and whether or not IIA Fru is fused to a BglG-like transcriptional regulator (Barabote and Saier 2005).

Further studies revealed that over evolutionary time, fusion and splicing of PTS proteins to give proteins of varying sizes and domain compositions have occurred frequently. Moreover, horizontal transfer of PTS genes accounts for the varied PTS protein compositions of closely related organisms. It appears that transporter gene transfer between bacteria has occurred with frequencies that are roughly inversely proportional to phylogenetic distance, and horizontal transfer between bacterial phyla has been surprisingly rare. Moreover, transfer between the three domains of life has almost never occurred. For example, genes encoding PTS proteins could not be identified in the archaeal or eukaryotic domains (Barabote and Saier 2005) while genes encoding members of the Mitochondrial Carrier family are not present in prokaryotes (unpublished observations). It is assumed that these two superfamilies of transporters evolved after the great division between bacteria, archaea, and eukaryotes. Finally, fusions of PTS domains to non-PTS enzymes and transport systems suggest novel PTS regulatory functions not yet recognized (Barabote and Saier 2005).

#### 4.8. Multidrug Resistance Pumps

The occurrence of MFS-type MDR efflux pumps in LAB is much higher than in most other bacteria as noted in Section 4.2. For example, LAB have over 60% of their MFS carriers functioning as MDR/hydrophobic compound exporters compared with 40%–50% for other bacteria (Table 4.1; Lorca et al. 2007), thus showing an unexpected trend. Moreover, in most LAB, MFS porters predominate: MFS (55%) > ABC (32%) > MOP (7%) > RND (4%) > DMT (2%). However, in *S. thermophilus*, the order is MOP (47%) > ABC (37%) > MFS (16%) > RND or DMT, and in *L. delbrueckii* and *L. gasserii*, these ratios are ABC (51%) > MFS (44%) > RND (<5%) > MOP or DMT (0%). It is therefore clear that while LAB exhibit unusual trends, relative to other bacteria, they sometimes exhibit considerable variation among themselves.

#### 4.9. Nutrient Transport in LAB

Three mechanisms account for sugar uptake in bacteria. In LAB, PTS permeases (52%) predominate over secondary carriers (34%), which predominate over primary active ABC transporters (15%). Gram (+) firmicute pathogens show similar proportions. However, in actinobacteria, the order is secondary carriers (50%) > ABC transporters (41%) > PTS group translocators (8%), and in select Gram (–) pathogens, the order is ABC (70%) > PTS (22%) > secondary carriers (8%). These relative numbers may reflect the modes of energy generation in the various groups of organisms under study (Paulsen et al. 1998, 2000; Lorca et al. 2007). It is also interesting to note that in contrast to most other groups of bacteria, LAB prefer mammal- or plant-derived glycosides and oligosaccharides to simple sugars (Lorca et al. 2007).

In virtually all groups of prokaryotes studied, ABC uptake porters of the PepT family (TC# 3.A.1.5) predominate (71%–86% for bacteria and nearly 100% for archaea) relative to all other types of peptide uptake systems. Secondary active transport of peptides is relatively rare. In LAB, the order of secondary carrier family representation is POT

(TC# 2.A.17; 16%) > OPT (TC# 2.A.67; 10%) > AbgT (2.A.68) > PAT of the MFS (TC# 2.A.1.25) or of PUP (TC# 9.A.18) (nearly 0% representation). This same trend is valid for other bacterial types although the percentages of these secondary carriers, relative to ABC transporter, are usually greater for LAB. However, Gram (–) bacteria exhibit appreciable representation of PAT and PUP family members that are largely absent from Gram (+) bacteria. Since ABC systems usually transport their substrates with higher affinities than secondary active transporters, these observations may reflect peptide concentrations in the environments where these organisms evolved.

#### 4.10. Conclusions and Perspectives

Transport proteins in LAB constitute 13%–19% of the total genome-encoded genes, in spite of the fact that the average value for most organisms is close to 10% (Lorca et al. 2007). About 5% of these transporters are low-specificity, bidirectional, channel proteins involved in adaptation to stress conditions. About 55% are for nutrient uptake while ~40% are for efflux. Specificities for uptake systems are amino acids > sugars > cations = anions > peptides. For exporters, the preferences are drugs >> peptides > macromolecules.

Most LAB have more secondary carriers than ATP-dependent, primary active transporters. However, in some LAB (e.g., *L. delbrueckii* and *S. thermophilus*), the situation is the reverse (Lorca et al. 2007). These observations must reflect the environmental niches and evolutionary histories that these organisms have experienced. They probably also reflect the environments these organisms prefer to inhabit today.

LAB have tremendous industrial importance for fermentation of food products, for production and spoilage of wine and beer, and for the purpose of promoting mammalian health (see Introduction). Based on the studies presented and discussed in this chapter, it was found that these organisms share some unusual but nearly universal characteristics. They all have mechanosensitive channels for osmotic adaptation, and they have disproportion-

ately large numbers of protective drug efflux pumps. They also have a preponderance of oligosaccharide and glycoside uptake porters relative to transporters that take up free sugars. These organisms largely lack electron carriers although a very limited array of them has been discovered (Vido et al. 2004).

Characteristically, LAB have substantial numbers of peptide uptake and efflux systems, used for nutrition, signaling, regulation, and biological warfare. All LAB secrete proteins via Sec/Oxa1 systems, but they lack the Sec auxiliary proteins, SecDF. Because of their rapid growth rates, they may possess compensatory proteins that fulfill the same illusive function(s) of SecDF. Moreover, all LAB seem to have competence-related and septal DNA translocation proteins although competencies for DNA uptake are not a demonstrated characteristic of these organisms. Finally, all LAB examined have at least one (and sometimes more) peptidoglycan (murein) precursor exporters.

Other strikingly unusual characteristics of LAB include (1) the virtual absence of RND-type exporters; (2) the poor representation of DMT and MOP-type carriers, especially for drugs and metabolites; (3) the preponderance of MFS-type drug exporters (>60% of all MFS carriers); (4) the unusual distribution of the three independently arising ABC-types of integral membrane proteins, (ABC1:ABC2:ABC3 = 1>>3>2 for LAB as compared with most other bacteria (1>2>3>) and archaea (2>>1>3)); (5) the preponderance of PTS group translocators over primary and secondary active transporters for sugars, although all types are represented; (6) the tremendous variability in PTS transporter representation in the different LAB examined; (7) the unusual representation of divalent cation carriers with the NRAMP and VIT families being used for uptake and the CDF family largely responsible for export; and (8) the surprising representation of eukaryotic-type Na<sup>+</sup> and H<sup>+</sup>-ATPases (TC families 3.A.3.1, 3, and 9) absent in most other bacteria including other firmicutes.

Why do LAB have proportions of transporters so different from those of other bacteria, and why do they have a rich supply of transporters, some of

which are normally found only in eukaryotes? Perhaps their complement of transporters reflects their unusual lifestyles in association with plants and animals. But why do they have distributions of transporter types that differ so much from other microbes, and sometimes from each other? The obvious generalized answer has to be EVOLUTION, but what specifically? There are probably multiple answers: (1) their reduced genome sizes may have allowed the retention of only the most important transporters for these organisms, coping in their individualistic niches; (2) their unique habitats reflecting unusual types of nutrient availability certainly played a role; (3) their apparent obsession with competition and protection, and their preoccupation with biological warfare against other microbes rather than eukaryotes must have been important; and (4) possibly a “desire” or need for communal life, dependent on intra- and interspecies communication also played a role. Their close mutually beneficial associations with eukaryotic animals and plants, for example, was probably important, particularly to facilitate horizontal gene transfer between the eukaryotic and bacterial domains.

## Note

This chapter in part reviews aspects of published work (Lorca et al. 2007); it also reports previously unpublished work reported in a plenary talk presented at the 9th Symposium on Lactic Acid Bacteria held on August 31–September 4, 2008 at Egmond Aan Zee in the Netherlands.

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## Chapter 5

# Applications of Lactic Acid Bacteria-Produced Bacteriocins

Barry Collins, Paul D. Cotter, Colin Hill, and R. Paul Ross

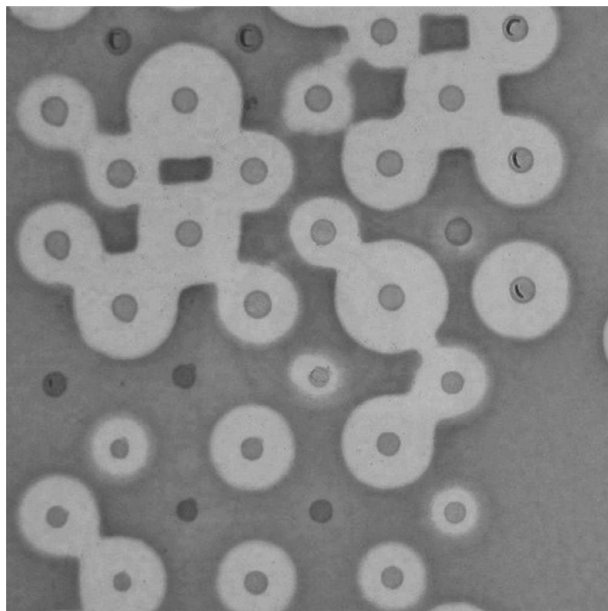
*Bacteriocins are antimicrobial peptides produced by a wide variety of bacteria, including the lactic acid bacteria (LAB), which are active against other Gram (+) bacteria. The fact that LAB bacteriocins frequently exhibit activity against significant spoilage and disease-causing bacteria means that there are also a broad number of ways in which they could be applied, for example, shelf-life extension, bio-preservation, control of the fermentation flora, and potentially as clinical antimicrobials. While there is obvious merit in identifying and investigating versatile broad-spectrum bacteriocins, there is also scope for the exploitation of those with a narrow spectrum for the targeting especially problematic species. The ways in which LAB bacteriocins have been applied and an assessment of their potential future applications will be reviewed here. In addition, as commercial interest in bacteriocins grows, it is becoming increasingly important to assess the possibility of the development of resistance to specific bacteriocins among target species. Thus a literature review to assess the likelihood of such an occurrence is also included.*

### 5.1. Introduction

Lactic acid bacteria (LAB) are a diverse and immensely useful group of bacteria that, while not adhering to a strict taxonomical grouping, are associated on the basis of shared properties (Pfeiler and Klaenhammer 2007). The most significant common trait is the production of lactic acid as a major or

sole product of fermentation. Other shared features of these Gram (+) microorganisms include a low GC content as well as being relatively acid tolerant, non-sporulating rods or cocci. Due to the production of lactic acid, LAB have historically been associated with the fermentation of food, and as a result many LAB (including *Lactococcus*, *Oenococcus*, *Lactobacillus*, *Leuconostoc*, *Pediococcus*, and some *Streptococcus* sp.) have a Generally Recognized as Safe status, although others, including species of *Enterococcus* and *Streptococcus*, are pathogenic in nature (Madigan et al. 2003). LAB grow anaerobically, although in many cases they are not sensitive to O<sub>2</sub>, and obtain energy exclusively from sugar metabolism, thereby restricting their presence to environments in which appropriate sugars are abundant. Many LAB, particularly those isolated from a protein-rich environment such as milk, have a limited biosynthetic ability and their complex nutritional requirements include amino acids, vitamins, purines, and pyrimidines.

As is the case with many other bacteria, bacteriocin production is common among the LAB (Fig. 5.1). Bacteriocins are ribosomally synthesized antimicrobial peptides produced by bacteria that are active against other bacteria. It has been noted that the activity of bacteriocins is frequently directed against bacteria that are related to the bacteriocin-producing strain or against bacteria found in similar environments. It has also been noted that some bacteriocins can also play a role in cell signaling. Microorganisms that produce bacteriocins also



**Figure 5.1.** Different bacteriocin-producing strains of bacteria overlayed with a bacteriocin-sensitive species. Antagonism between bacteriocin producers and the indicator is seen as zones of clearing where the sensitive species do not grow. Bactericidal effect can be species-specific.

possess immunity mechanisms to confer self-protection, that is, to protect bacteriocin producers from committing “suicide” (Cotter et al. 2005; Draper et al. 2008). It has been estimated that between 30% and 99% of all bacteria and Archaea produce bacteriocins; their production by LAB is very significant from the point of view of their potential applications in food systems and thus, unsurprisingly, these have been most extensively investigated.

Of the LAB bacteriocins identified to date, nisin, the original lantibiotic (or class I) bacteriocin, has been the most extensively investigated and remains the only bacteriocin to have been approved for use by the European Union and the Food and Drug administration (FDA; Guinane et al. 2005). Several variants of nisin, which is 34 amino acids long and has a broad-spectrum of activity against strains of Gram (+) bacteria, are produced by *Lactococcus lactis* or *Streptococcus uberis* (Mulders et al. 1991; Wirawan et al. 2006). Nisin has been used in a variety of applications because of its broad-spectrum of activity and, because it is easily broken

down by gut proteases, it does not cause harm to the gut microflora. Nisin has commonly been used in processed cheese, various pasteurized dairy products, and canned vegetables; many additional food-related uses have also been proposed. There has also been much interest in using nisin for the treatment or prevention of human diseases and in veterinary products (Delves-Broughton et al. 1996). It is important to also note that, although as yet unexploited in the majority of cases, many other bacteriocins also offer potential benefits to producers and consumers of food. These benefits primarily involve a reduced requirement for chemical or physical processes for the preservation of food. These goals can be facilitated through the use of bacteriocins as concentrated preparations, as crude fermentates, or through the incorporation of live bacteriocin-producing strain(s), either through direct addition to the food or in an immobilized form on packaging. Bacteriocins can, of course, also be used in conjunction with other factors such as high pressure or pulse electric fields, to achieve more effective preservation of foods

(Galvez et al. 2007). In addition to roles in the food industry, the potential of a number of LAB bacteriocins other than nisin have also been investigated with a view to potential clinical uses; for example, the lantibiotic lactacin 3147 has been shown to be effective at killing drug-resistant pathogens such as methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci (VRE), and *Clostridium difficile* (Galvin et al. 1999; Rea et al. 2007), while other studies have demonstrated the ability of other lantibiotics to control *Streptococcus pneumoniae* and MRSA infections in mice (Niu and Neu 1991; Goldstein et al. 1998) or to prevent tooth decay and gingivitis (Howell et al. 1993; McConville 1995). Bacteriocin-containing products have also been shown to be of benefit in treating bovine mastitis (Ryan et al. 1999) and even in throat sprays for improving bad breath (Tagg 2004).

## 5.2. Bacteriocins of LAB

Although a number of different classification systems have been proposed for LAB bacteriocins (Klaenhammer 1993; Nes et al. 1996), for the purpose of this review we have adopted a relatively recent approach that reflects the fact that most bacteriocins can be grouped into two classes with relative ease, that is, the lanthionine-containing bacteriocins or lantibiotics (class I) and the largely unmodified linear peptide antimicrobials (class II; Cotter et al. 2005).

### 5.2.1. Class I Bacteriocins

The lanthionine-containing bacteriocins or lantibiotics consist of small, heat stable peptides (19–39 amino acids in length) that contain unusual residues, including lanthionines (Lan) and/or  $\beta$ -methylanthionines (meLan; Cotter et al. 2005; Table 5.1). These unusual residues are introduced as a consequence of extensive posttranslational modifications. These modifications result primarily from the dehydration of serine (Ser) and threonine (Thr) residues to 2,3-dehydroalanine (Dha) and (Z)-2,3-dehydrobutyrine (Dhb), respectively. Neighboring cysteines (Cys) can become covalently linked to

**Table 5.1.** Lantibiotics of lactic acid bacteria.

Lantibiotic	Producing strain	Reference
Nisin A	<i>L. lactis</i>	Gross and Morell (1971)
Nisin Z	<i>L. lactis</i>	Mulders et al. (1991)
Nisin F	<i>L. lactis</i>	de Kwaadsteniet et al. (2008)
Nisin U	<i>Strep. uberis</i>	Wirawan et al. (2006)
Nisin U2	<i>Strep. uberis</i>	Wirawan et al. (2006)
Nisin Q	<i>L. lactis</i>	Zendo et al. (2003)
Mutacin	<i>Strep. mutans</i>	Mota-Meira et al. (1997)
B-NY266		
Mutacin 1140	<i>Strep. mutans</i>	Hillman et al. (1998)
Mutacin I	<i>Strep. mutans</i>	Tsang et al. (2005)
Streptin	<i>Strep. pyogenes</i>	Wescombe and Tagg (2003)
Macedocin	<i>Strep. macedonicus</i>	Georgalaki et al. (2002)
Lactacin 481	<i>L. lactis</i>	Piard et al. (1993)
Mutacin K8	<i>Strep. mutans</i>	Robson et al. (2007)
Mutacin II	<i>Strep. mutans</i>	Chikindas et al. (1995)
Streptococcin A-FF22	<i>Strep. pyogenes</i>	Tagg and Wannamaker (1978)
Salivaricin A	<i>Strep. salivarius</i>	Ross et al. (1993)
Salivaricin A1	<i>Strep. pyogenes</i>	Wescombe et al. (2006)
Salivaricin A2	<i>Strep. salivarius</i>	Wescombe et al. (2006)
Salivaricin A3	<i>Strep. salivarius</i>	Wescombe et al. (2006)
Salivaricin A4	<i>Strep. salivarius</i>	Wescombe et al. (2006)
Salivaricin A5	<i>Strep. salivarius</i>	Wescombe et al. (2006)
Salivaricin B	<i>Strep. salivarius</i>	Hyink et al. (2007)
Plantaricin C	<i>Lact. plantarum</i>	Turner et al. (1999)
Lactacin 3147	<i>L. lactis</i>	Ryan et al. (1996)
Lactacin J46	<i>L. lactis</i>	Huot et al. (1996)
SmbB	<i>Strep. mutans</i>	Yonezawa and Kuramitsu (2005)
BhtA	<i>Strep. rattus</i>	Hyink et al. (2005)
Lactosin S	<i>Lact. sake</i>	Mortvedt et al. (1991)
Plantaricin W	<i>Lact. plantarum</i>	Holo et al. (2001)
Bovicin HJ50	<i>Strep. bovis</i>	Xiao et al. (2004)
Bovicin HC5	<i>Strep. bovis</i>	Mantovani et al. (2002)
Cytolysin	<i>Ent. faecalis</i>	Gilmore et al. (1996)

these Dha or Dhb residues, resulting in the formation of Lan or meLan bridges, respectively (Willey and van der Donk 2007). These intramolecular bridges give lantibiotics their distinctive ring structures. Finally, the modified peptide is exported and the 23–59 amino acid leader sequence is proteolytically removed (Xie and van der Donk 2004). Additional, less typical, modified residues are occasionally observed in lantibiotics and in total at least

15 different posttranslational modifications have been identified. The extent to which such modifications occur varies with lactocin S (24% modified residues) and microbisporicin (58% modified residues) representing extreme examples (Gilmore et al. 1996; Castiglione et al. 2008). To add to this complexity, a number of two peptide lantibiotics have been identified. These are lantibiotics that function maximally as a result of a synergistic interaction between two lantibiotic peptides. A number of these two-component systems (i.e., lactacin 3147, staphylococcin C55, plantaricin W, Smb, BHT-A, and haloduracin) are very closely related (Lawton et al. 2007); however, the enterococcal two-peptide lantibiotic cytolysin is quite different and in fact functions as a virulence factor in that pathogen.

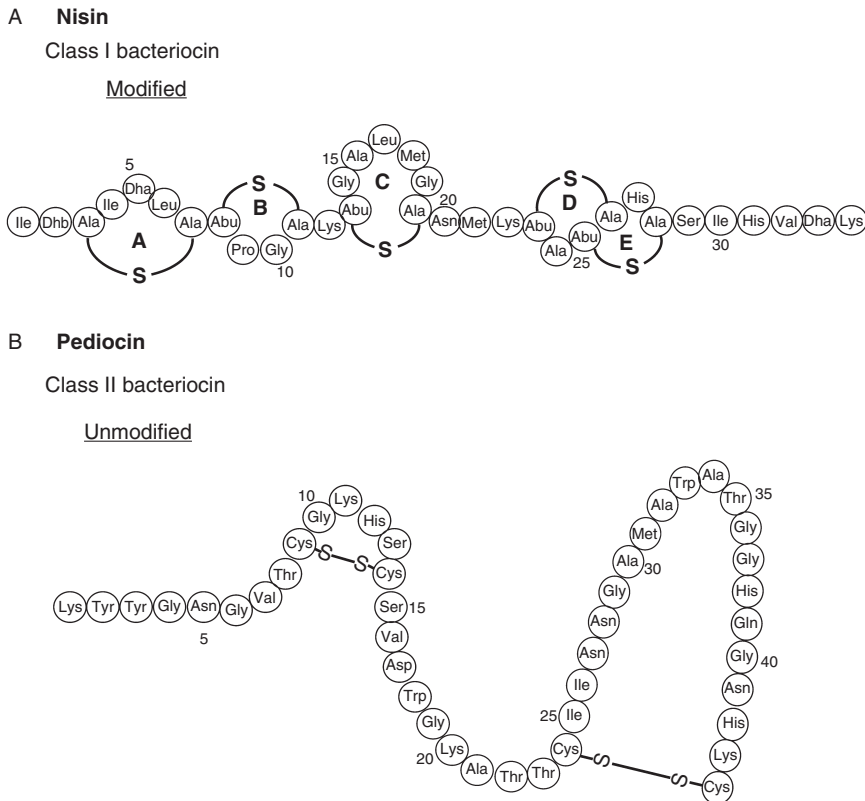
Lantibiotics can be further subdivided into three subclasses depending on whether they have antimicrobial activity and the mechanisms by which the peptide gains maturity. This type of scheme includes most peptides and leaves scope for the inclusion of peptides that have yet to be discovered. The modification method is the key difference between subclass I, subclass II, and subclass III lantibiotics. Analysis of the leader sequences of the different types of lantibiotics usually indicates the type of modification method that will be employed. When the leader sequences are compared, two conserved sequences have been identified. Lantibiotics with the conserved FNLD sequence between position -20 and -15 are posttranslationally modified by a two-enzyme system involving LanB and LanC, responsible for dehydration and cyclization, respectively. A generic designation *lan* is used to describe the genes of the lantibiotic gene clusters with, for example, *lanA* being used to denote the gene encoding the structural peptide. Subclass II peptides containing a characteristic "GG" or "GA" cleavage site have only one modification enzyme (generically termed LanM), which performs both the dehydrating and cyclizing roles (Chatterjee et al. 2005). Subclass III lantibiotics are lanthionine-containing peptides that are structurally similar to conventional lantibiotics but do not have significant antimicrobial properties; they are employed in a different role by

the producing microorganism. Very few of these peptides have been described to date. The putative modification enzymes of these peptides bear homology to the C-terminal cyclization domain of LanM enzymes but lack associated essential zinc ligands and thus may be modified in a unique way. To date, no species of LAB has been discovered that produces any of these compounds (Willey and van der Donk 2007). An alternative, not necessarily mutually exclusive, scheme for the subclassification of lantibiotics has been proposed whereby the peptides have been divided on the basis of similarity between the structural peptides, with each subclass named on the basis of the name of the prototypical lantibiotic. Of these, the nisin, epidermin, streptin, lactacin 481, mersacidin, and LtnA2 subclasses all contain lantibiotics produced by members of the LAB (Twomey et al. 2002; Field et al. 2007).

Following complete modification lantibiotic peptides are exported from the cell by dedicated transporters. Subclass I peptides are expelled by an ATP-binding cassette (ABC) transporter, LanT, while LanP, a subtilisin-like serine protease, cleaves the accompanying leader peptide. The secretion and processing of subclass II lantibiotics is performed by a single multifunctional protein with a conserved N-terminal cysteine protease domain, which is also referred to as LanT despite differing from the subclass I transporters of the same name. Thus far, two peptide lantibiotics have all been representatives of subclass II lantibiotics (Lawton et al. 2007).

One of the key characteristics of bacteriocin-producing bacteria is the production of proteins that protect producers against the action of their own bacteriocin. This phenomenon is known as bacteriocin immunity. Lantibiotic-producing microorganisms rely on a dedicated immunity protein LanI and/or an ABC transporter encoded by *lanFE(G)*. The mode of action of at least some LanI proteins is to bind at the membrane-cell interface, obscuring the target site and therefore protecting the cell from its own lantibiotic (Hoffmann et al. 2004). Alternatively, or in addition, the LanFE(G) ABC transporter expels the antimicrobial peptide from the cell into the extracellular environment (Stein et al. 2003; Draper et al. 2008).





**Figure 5.2.** Prototypical bacteriocins of the LAB. A. Nisin, the typical lantibiotic has the characteristic lantibiotic lanthionine bridges, resulting in the formation of ring structures; B. Pediocin, the prototype class II bacteriocin, has an unmodified structure.

As noted above, the prototypic lantibiotic nisin is the prototype of the subclass I lantibiotics (Fig. 5.2). The genes responsible for nisin production, processing, immunity, and regulation are located on a large conjugative transposon and are organized in four operons, that is, *nisABTCIPRK*, *nisI*, *nisRK*, and *nisFEG*. The nisin equivalent of LanA (NisA) consists of a 57-residue pre-peptide that is ribosomally synthesized before undergoing extensive posttranslational modification by the processing machinery encoded by *nisB* and *nisC* (Rink et al. 2007). Lacticin 481 is a typical class II lantibiotic, the genetic determinants of which are encoded by a single six-gene operon, *lctAMTFEG*, located on a 70-kb plasmid. As lacticin 481 is a member of subclass II, it utilizes the alternative, LanM-

dependent, modification mechanism (Dufour et al. 2007).

Though the lantibiotics are predominantly active against Gram (+) bacteria, limited activity against Gram (-) organisms has been noted which, in general, requires prior disruption of the outer membrane of the Gram (-) cell envelope by another agent (Hoffmann et al. 2002). Many lantibiotics, including nisin, exhibit dual modes of action, both facilitated by the target lipid II. Lipid II is an essential intermediate in cell wall synthesis, and the nisin:lipid II complex both inhibits normal peptidoglycan formation and also facilitates the assembly of pores in the cell membrane resulting in the loss of membrane potential and the efflux of nutrients (Wiedemann et al. 2001; van Heusden et al. 2002;

Wiedemann et al. 2004). While binding to lipid II is a key step in the activities of a number of lantibiotics (Wiedemann et al. 2006a, 2006b), on some occasions the inhibition of peptidoglycan synthesis is the sole means via which the peptide functions (Dufour et al. 2007).

### 5.2.2. Class II Bacteriocins

The class II bacteriocins are small, heat-stable, non-lanthionine-containing peptides that are not subject to extensive posttranslational modification. Many class II bacteriocins are active in the nanomolar range and pore formation is the most common mode of action. The heterogeneous nature of these peptides makes classification difficult. Cotter et al. (2005) proposed four subclasses consisting of the pediocin-like (subclass a bacteriocins; Fig. 5.2), two-peptide (subclass b bacteriocins), cyclic (subclass c), and non-pediocin single peptide linear bacteriocins (subclass d).

Class IIa bacteriocins, which have been identified in a wide range of LAB, have a narrow spectrum of activity but are particularly potent against *Listeria monocytogenes*. They range in size from 27 to 48 amino acids and are distinguished by the presence of a YGNGVXCXXXXCXV motif toward the N-terminal, which includes two cysteines that form a disulphide bridge. Class IIa pre-peptides contain an N-terminal leader sequence that is required for interaction with a dedicated transporter and ensures that the pre-peptide remains inactive until secretion from the cell. The leader generally consists of 15–30 residues and is most frequently removed during export by cleavage at the C-terminal side of a double-glycine motif (Havarstein et al. 1995). A limited number of peptides have a Sec-type leader sequence resulting in translocation via the general Sec pathway present in many bacteria (Cintas et al. 1997; Kalmokoff et al. 2001). The N-terminal domain of the active peptide adopts an S-shaped three-stranded anti-parallel  $\beta$ -sheet conformation that is stabilized by a disulfide bridge and this region is responsible for initiating binding to the target cell (Fimland et al. 2005). The C-terminal domains, which are located after a central “hinge” region, are less con-

served. The C-terminal domain has been used to further divide the class IIa bacteriocins into three further subgroups (Johnsen et al. 2005b). The peptides of subgroup 2 tend to be shorter than those within subgroups 1 and 3 due to a reduced C-terminal section whereas peptides in subgroup 3 are distinguished by the absence of a cysteine and/or a tryptophan that stabilizes the hairpin found at the C-terminal end of subgroup 1 and 2 peptides. It is thought that the hinge region endows the peptide with additional flexibility, allowing the C-terminal domain to dip into the hydrophobic region of the membrane, resulting in the leakage of cell constituents (Uteng et al. 2003; Fimland et al. 2006). In studies where hybrid peptides were created by joining N- and C-terminal regions from different pediocin-like peptides, it was apparent that the C-terminal domain contributes greatly to determining the specificity of pediocin-like bacteriocins (Johnsen et al. 2005b). The mechanisms for target cell recognition and producer cell immunity have been the subject of intense investigation (Dalet et al. 2001; Gravesen et al. 2002b; Ramnath et al. 2004) and it has been established that components of the mannose phosphotransferase system are used as a target or receptor for lactococcin A and other class II bacteriocins (Diep et al. 2007).

While in the majority of cases the genes encoding class IIa bacteriocins are located on plasmids, there are some exceptions, such as enterocin A and sakacin P, where these genes are chromosomally located. The genes can be organized in a single or multiple operons (Drider et al. 2006). The four genes associated with production of immunity to plantaricin 423, pediocin PA-1 and coagulin are located in one operon whereas the genes involved in the production of sakacin P, divergicin V41, and enterocin A are spread over several operons containing the structural and immunity genes, secretion genes, and regulatory genes (Ennahar et al. 2000). The aforementioned “four-gene” class IIa bacteriocins consist of genes encoding the structural pre-peptide, an immunity protein (often also a peptide) required for self-protection, an ABC transporter that transports the bacteriocin across the membrane and removes the associated leader sequence, and an

accessory protein that is thought to be linked to secretion (Fimland et al. 2005). While the majority of such peptides are exported through recognition of the specific leader region by a dedicated ABC transporter (Havarstein et al. 1995), as noted above some bacteriocins contain leaders of the “Sec type,” which are cleaved during bacteriocin secretion by the multifunctional bacterial Sec pathway (Sanchez et al. 2008). Enterocin P, bacteriocin 31, and bacteriocin T8 are all class IIa Sec-dependent bacteriocins (De Kwaadsteniet et al. 2006).

The Class IIb bacteriocins are two-peptide bacteriocins that, like their lantibiotic counterparts, exhibit synergistic (rather than additive) activity when the two complementary peptides are combined. These bacteriocins are also distinguished from other bacteriocins that function synergistically by the fact that the structural genes for both peptides are found within the same operon (Eijsink et al. 2002). It is common for one or both of the peptides to lack activity when assessed individually but to have potent activity when used in combination with its partner peptide. Genetically the two-component bacteriocins have at least five genes, in one or two operons, which encode two structural peptides, an immunity protein, an ABC transporter, and an accessory protein that is thought to be linked to transport. All of the two component bacteriocins characterized have a 15- to 30-residue N-terminal leader sequence, of the double glycine type. This is removed during export by the ABC transporter. Production of some members of the class IIb bacteriocins is regulated by a regulatory mechanism consisting of a three-component signal transduction system. These systems are comprised of a secreted peptide pheromone, a membrane-associated histidine protein kinase sensor, and an intracellular response regulator (Oppeggard et al. 2007). The remaining groups of class II bacteriocins are class IIc that are cyclic peptides, that is, peptides in which the N- and C-termini are covalently linked resulting in a cyclic structure, and the one peptide non-pediocin linear peptides that comprise class IId.

As with the lantibiotics, producers of class IIa bacteriocins must protect themselves via a dedicated immunity mechanism (Johnsen et al. 2005b; Van

Reenen et al. 2006). The most common method of self-protection among producers of class IIa, class IIb, or class IId bacteriocins involves a single immunity protein whereas class IIc bacteriocins rely on an ABC transporter (Cotter et al. 2005). A comparison of class IIa immunity proteins has shown that they can vary dramatically, with anything from 5%–85% similarity (Eijsink et al. 1998). Although the protection provided by these peptides is in general very specific, it is unsurprising, given the occasional high levels of homology, that some class IIa immunity proteins have been shown to confer cross-resistance. These immunity proteins range in size from 81 to 115 amino acids and can be arranged into three groups (group A–C) based on sequence similarity. The structure of four class II immunity proteins have been determined, group A (EntA-im and PedB), group B (PisI), and group C (ImB2; Sprules et al. 2004; Johnsen et al. 2005a; Kim et al. 2007; Martin-Visscher et al. 2008). These proteins fold into a globular conformation in aqueous solutions and contain an antiparallel four-helix bundle, which is a defining characteristic of class IIa immunity proteins (Martin-Visscher et al. 2008). In general, the immunity genes associated with these bacteriocins reside on the same operon as the structural genes with co-expression ensuring that bacteriocin and immunity protein production is coordinated. It has also been observed that bacteria can be more sensitive to other bacteriocins when they are not producing their own bacteriocin (Drider et al. 2006).

With regard to mode of action, most class II bacteriocins are thought to form pores in the bacterial membrane that result in leakage of key constituents from the cell, disruption of the proton motive force, and ATP depletion. As much as the formation of pores is common among bacteriocins, the conductivity, size, and stability of pores vary greatly (Eijsink et al. 2002). As noted above, the mannose phosphotransferase system (PTS) has been identified as the receptor for class IIa bacteriocins (Diep et al. 2007), while recently lactococcin 972 (class IId) has been shown to be the first example of a non-lantibiotic bacteriocin that interacts with lipid II (Martinez et al. 2008a).

### 5.3. Applications

#### 5.3.1. Applications of Class I Bacteriocins

One of the most important roles of lantibiotics is the inhibition of food-borne pathogenic bacteria such as *L. monocytogenes*, *S. aureus*, *Clostridium botulinum*, *Clostridium perfringens*, *Bacillus cereus*, and others (Brotz et al. 1995; Guerlava et al. 1998; Pol et al. 2000; Rodgers et al. 2003; Nguyen et al. 2008). Lantibiotics can also inhibit spoilage microorganisms that can be very costly to the food industry, for example, *Clostridium tyrobutyricum* and *B. cereus* (Rilla et al. 2003; de Carvalho et al. 2007). Nisin is the main lantibiotic applied by industry to date. It has been commercially available since 1953 and in 1969 gained approval from the joint Food and Agriculture Organization/World Health Organization expert committee on additives. In 1983 it was added to the European food additive list and in 1988 it was approved for use by the FDA in processed cheese spreads (Cotter et al. 2005; Delves-Broughton 2005). Thus, nisin is the bacteriocin most frequently utilized to control spoilage and pathogenic bacteria. Six natural variants of nisin have been discovered to date. The most common nisin variants are nisin A and Z although other variants have been characterized, including nisin Q, U, U2, and the newest variant nisin F. Nisin A, nisin Z, nisin F, and nisin Q are produced by *L. lactis*. Nisin U and nisin U2 are produced by *Strep. uberis* (De Kwaadsteniet et al. 2008; Lubelski et al. 2008). Of these only nisin A is available commercially, albeit in various forms, for example, as Nisaplin®, a product containing 2.5% nisin A (with the remainder of the powder being milk solids derived from the fermentation of a modified milk medium by a nisin-producing strain of *L. lactis*). Even though nisin is the only lantibiotic that is currently used in this manner (Delves-Broughton 2005), other lantibiotics such as lactacin 3147 and variacin have displayed great potential when similarly prepared and used as a sole preservative (Morgan et al. 2001; O'Mahony et al. 2001) or as part of a hurdle approach in food processing. Hurdle technology involves the use of multiple antimicrobial factors to greatly increase the efficacy of the elimination of microorganisms, and bacteriocins

have been utilized in combination with heat, modified atmosphere packaging, pulsed electric fields, high hydrostatic pressure, and other antimicrobial compounds in this way (Nilsson et al. 1997; Ueckert et al. 1998; Pol and Smid 1999; Dutreux et al. 2000; Morgan et al. 2000). The hurdle approach has also provided a means via which previously insensitive Gram (–) bacteria can be sensitized to nisin; it is possible to render *E. coli* nisin sensitive when used in conjunction with high-pressure homogenization (Diels et al. 2005).

An alternative means of utilizing lantibiotics in food as preservatives has involved the use of lantibiotic-producing starter cultures during food fermentations. Bacteriocins can also be incorporated into fermented foods to control the growth of non-starter LAB (NSLAB) and to bring about the lysis of starters, NSLAB, and adjunct strains during cheese making, thereby releasing enzymes into the cheese that result in the breakdown of low molecular weight peptides and free amino acids and contributing to quality, texture, and flavor (O'Sullivan et al. 2002, 2003a). It should also be noted that in some instances it is sufficient to apply the lantibiotic-producing strain to the surface of a food, for example, a mold-ripened cheese, in order to provide protection against pathogenic bacteria (O'Sullivan et al. 2006).

As noted in the introduction, lantibiotics have also been shown to have applications in the field of human health. With respect to oral health, a *Streptococcus salivarius* producing the lantibiotic salivaricin A has been shown to reduce the bacteria implicated in halitosis (Burton et al. 2006), while milk products supplemented with a salivaricin A-producing strain have been shown to help protect against infection with *Streptococcus pyogenes*, which is responsible for oral pharyngitis (Dierksen et al. 2007). Furthermore, strains of *Streptococcus mutans*, which produce the lantibiotic mutacin 1140 and which have been mutated to diminish their acidogenic phenotype, have been successfully employed to competitively inhibit plaque-forming *S. mutans*. The 1140-producing strain of bacteria has been modified to be easily eliminated from the human host in case of adverse reactions (Hillman

et al. 2007). In addition to eliminating a wide range of pathogenic bacteria, it is worth noting that certain lantibiotics are capable of killing so-called “super bugs,” MRSA, VRE, and *C. difficile* (Galvin et al. 1999; Kruszewska et al. 2004; Rea et al. 2007).

The area of sexual health and contraception could also benefit from recent studies that show that nisin and lacticin 3147 are capable of killing sperm from various animal species (Reddy et al. 2004; Silkin et al. 2008). When this is combined with their existing antimicrobial properties, the potential of these peptides as novel anti-sexually transmitted infection contraceptive additives is evident. The main application considered from the veterinary standpoint is the utilization of bacteriocins to combat mastitis, the most costly disease to the dairy industry worldwide. Lacticin 3147 has been investigated to determine its efficacy against the main mastitis pathogens (Ryan et al. 1998; Ryan et al. 1999). It has also been used as an additive in bovine mastitis teat seals and has improved the antimicrobial capacity of this product (Twomey et al. 2000; Crispie et al. 2005).

### 5.3.2. Applications of Class II Bacteriocins

From a commercial perspective, the most important class II bacteriocin is pediocin PA-1, which is present in the commercial product Alta™ 2341 (Quest International, Hoffman Estates, IL). This fermentate is used as a shelf-life extender in a number of foods and as a protectant against *L. monocytogenes* in ready-to-eat meat products (Rodriguez et al. 2002). The class IIa bacteriocins are ideally suited to the protection of fermented foodstuff from *L. monocytogenes* in that they successfully antagonize *L. monocytogenes* without affecting the lactococci involved in fermentative processes (Eijsink et al. 1998). Pediocin PA-1 has also been employed in food in other ways. When used in conjunction with other antimicrobials it efficiently removes viable *L. monocytogenes* from the surface of beef frankfurters as well as from freshly cut produce (Uhart et al. 2004; Bari et al. 2005). Producers of PA-1 can also be employed directly; for example,

surface spraying of a cell suspension of a PA-1-producing *Lactobacillus plantarum* can protect cheese from *L. monocytogenes* contamination (Ennahar et al. 1996).

### 5.3.3. Lantibiotic Bioengineering

Due to the gene-encoded nature of bacteriocins, there are potentially significant benefits to employing modern cutting-edge bioengineering to progress the field beyond the scope of traditional peptide discovery, description, and production. One particular outlet for bioengineering in the lantibiotic field involves the creation of strains that produce ever greater quantities of lantibiotic peptide. The introduction of additional copies of genes encoding nisin regulators had such an impact on nisin production (Cheigh et al. 2005), and for lacticin 3147 overproduction was achieved through the addition of extra copies of the biosynthetic/production machinery and regulatory genes. Additional copies of the structural genes were not essential for this approach to be successful (Cotter et al. 2006b). Another strategy to improve the efficacy of lantibiotic-producing strains is to manipulate specific strains such that more than one antimicrobial is produced through the conjugation of multiple large bacteriocin-encoding plasmids into a single strain. Such strains have been found to be more effective at eliminating problematic species than the parent strains (O’Sullivan et al. 2003b). It is also possible to achieve this goal through the amplification and cloning of lantibiotic-encoding genes into shuttle vectors and heterologous production in other strains. Such an approach was used to facilitate the production of lacticin 3147 by an *Enterococcus* host (Ryan et al. 2001).

The bioengineering of existing and novel peptides could also lead to the generation of lantibiotics with improved potency and/or suitability for specific applications. Before employing mutagenesis to bioengineer these peptides it is important to know which amino acids are amenable, and which are intolerant, to change. One approach that has been taken to address this issue in lacticin 3147 relied on



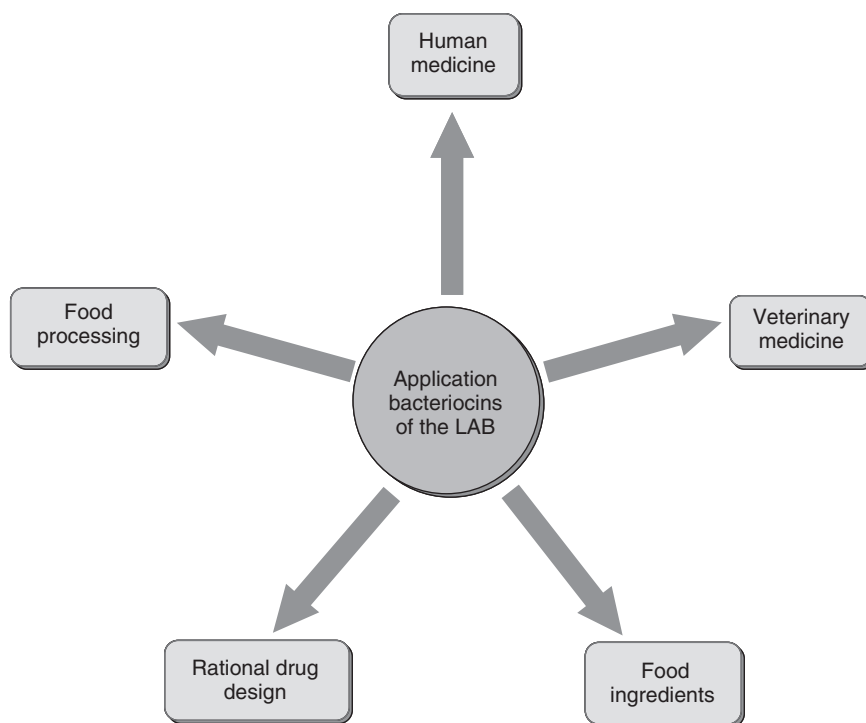
alanine-scanning mutagenesis whereby all residues are converted in turn to an alanine (or a glycine should an alanine be naturally present; Cotter et al. 2006a). This study revealed the identity of essential and nonessential residues and will be of great use with respect to the further rational drug design of this lantibiotic.

A number of other studies have provided insights into the structure/function relationships of specific lantibiotics and have, for example, been crucial in revealing the importance within the hinge region (a three-amino acid stretch that links the N-terminal lipid II-binding and C-terminal pore-forming domains) of nisin and related peptides. The importance of this region was apparent when manipulation of the hinge region in mutacin II resulted in a loss of activity (Chen et al. 1998) while discrete alterations in the hinge region of nisin Z resulted in mutants that had improved stability at elevated temperatures and neutral or basic pH (Yuan et al. 2004). Recently, the most comprehensive manipulation of the nisin hinge region to date has proved particularly revealing. This work was prompted by the discovery that the alteration leading to the creation of a nisin variant with enhanced activity against *Streptococcus agalactiae* could be traced as a change within the hinge region (K22T). Subsequently a saturation mutagenesis approach was adopted to further analyze this region, and variants with enhanced activity against *S. aureus* and *L. monocytogenes* were discovered. This discovery heralds an exciting era in lantibiotic engineering as it is now evident that it is possible to generate nisin variants with increased activity against significant Gram (+) pathogens (Field et al. 2008). In addition to improving activity or inhibitory spectrum, it has been shown that it is also possible to develop peptides that are enhanced with respect to other characteristics. This fact was perhaps best illustrated by nisin Z studies that showed that solubility and stability can be significantly improved by peptide engineering without dramatically reducing specific activity (Rollema et al. 1995). Given the existence of similar systems to facilitate the bioengineering of non-LAB lantibiotics, for example,

subtilin, Pep5, and mersacidin (Liu and Hansen 1992; Bierbaum et al. 1994; Szekat et al. 2003; Heinzmann et al. 2006), it is apparent that we may be entering an exciting new era of lantibiotic bioengineering.

While the approaches described above rely on the subtle alteration of natural lantibiotic producers, it is possible to more dramatically alter lantibiotic and non-lantibiotic peptides by altering existing, or introducing new, posttranslational modifications through the application of lantibiotic biosynthetic enzymes (and specific LanM and LanB/C proteins in particular). The ability of such enzymes to bring about cyclization is significant as such structures have been shown to protect peptides against peptidases and proteases, a property which is particularly useful from a drug design standpoint (Li and Roller 2002). Using such an approach the NisC enzyme has been utilized to cyclize and, as a result stabilize, non-lantibiotic peptides (Rink et al. 2007). NisB has also been used to introduce dehydro residues and facilitate the formation of thioether bridges into various peptides (Klusens et al. 2005). The LanM proteins such as LctM (the lactacin 481 modification protein) can be similarly exploited (Chatterjee et al. 2006).

Of course bioengineering of bacteriocins is not limited to lantibiotics. Much work has been carried out with the class IIa bacteriocins to determine the structure-function relationships. However, though the variants generated in these studies are useful from an academic standpoint, none of them display increased activity against pertinent microorganisms (Fleury et al. 1996; Fimland et al. 2002, 2006; Kazazic et al. 2002). Similarly, stability has been investigated, and although it was established that it is possible to generate more stable variants of pediocin PA-1, yet again no increases in activity were apparent (Johnsen et al. 2000). Recently mutants of pediocin PA-1 have been generated using a technique known as DNA shuffling. Some of these variants have shown increased activity against important food-spoilage organisms, possibly heralding a future application as food additives (Tominaga and Hatakeyama 2007; Fig. 5.3).



**Figure 5.3.** Applications of the bacteriocins of LAB. An example of the role bacteriocins can play in food processing is when they are applied as bacteriocin washes for treating prewashed vegetables, where they are effective at reducing *listerial* load (Allende et al. 2007). Bacteriocins are commonly used as food ingredients where they are added to foodstuff to protect against spoilage and pathogenic organisms (Cleveland et al. 2001). In the field of human medicine, bacteriocins have been shown to be useful against strains of the important human pathogens, for example, MRSA and VRE (Lawton et al. 2007). Veterinary products containing bacteriocins are effective at inhibiting important bovine mastitis pathogens (Ryan et al. 1999). An expanding area of interest is the utilization of bacteriocins in rational drug design; a vancomycin-nisin hybrid that has activity against VRE has been developed (Arnusch et al. 2008).

## 5.4. Bacteriocin Resistance

The problem of antibiotic resistance is a substantial challenge facing the global community at large today. It is estimated that approximately 70% of pathogenic bacteria are resistant to a least one form of antibiotic; some strains are even resistant to all common antibiotics and require the use of new and experimental compounds. As much as bacteriocins are being considered as alternatives to antibiotics in certain settings, it is important to determine whether bacteria readily develop resistance to bacteriocins and the means by which this could occur. It is thought that there are at least four processes involved

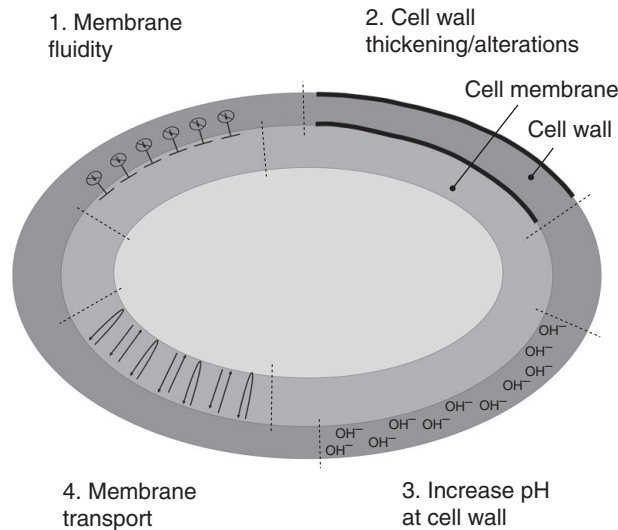
in nisin resistance, that is, preventing the bacteriocin from reaching the cytoplasmic membrane, reducing the acidity of the extracellular medium thereby stimulating binding of the bacteriocin to the cell wall, preventing the insertion of the bacteriocin into the membrane, and transporting the peptide out of the membrane (Kramer et al. 2006). It should however also be noted that bacteria differ greatly in their natural sensitivity to different bacteriocins as a consequence of differences in cell envelope composition. It has been demonstrated that in closely related strains of *L. lactis*, the level of sensitivity to specific bacteriocins such as nisin and pediocin PA-1 can vary by 100- to 1000-fold (Bennik et al. 1997) and

thus it is possible for bacteria living in similar environments to have dramatically different sensitivities to the same bacteriocins. Whereas up to 8% of wild-type strains of *L. monocytogenes* appear to be naturally resistant to pediocin-like bacteriocins and natural variation in nisin sensitivity also exists, it is also apparent that resistance can develop upon exposure to the bacteriocin. For example, spontaneous resistance emerges among previously nisin-sensitive *L. monocytogenes* isolates at a frequency of between  $10^{-7}$  and  $10^{-2}$  upon exposure to nisin and at a frequency of  $10^{-6}$  upon exposure to pediocin PA-1 (Gravesen et al. 2002a). This rate is quite high when one considers that, in general, upon exposure to conventional antibiotics, resistance develops at a rate in the region of  $10^{-7}$  to  $10^{-9}$ . It should be noted however that resistance to nisin and pediocin PA-1 can also have an associated fitness cost (Gravesen et al. 2002a). Furthermore, there are a number of bacteriocins to which bacteria do not readily develop resistance; for example, when *L. lactis* IL1403 was challenged with lacticin 3147, only a slight decrease in sensitivity was observed and this only occurred at a low frequency of  $10^{-8}$  to  $10^{-9}$  (Guinane et al. 2006). A phenomenon to note in bacteriocin resistance is the development of cross resistance and of particular interest is cross-resistance among the differing classes of bacteriocins (Naghmouchi et al. 2007). Bacteriocin resistance has also been associated with an increased bacteriophage resistance (Maisnier-Patin and Richard 1996; Martinez et al. 2008b) but frequently also results in the strain being more antibiotic-sensitive than its wild-type counterpart (Gravesen et al. 2001; Mantovani and Russell 2001; Cotter et al. 2002; Martinez and Rodriguez 2005; Guinane et al. 2006; Naghmouchi et al. 2007).

Alterations within the cell envelope are repeatedly seen in bacteria with altered resistance to nisin. Cell-wall thickening at the septum appears beneficial to nisin-resistant *L. lactis*. As lipid II gathers at the cell-division site, it is believed that this thickening shields the membrane and lipid II, thereby protecting the cell against nisin (Kramer et al. 2008). It should be noted, however, that despite acting as the nisin receptor, lipid II levels do not dictate nisin

sensitivity (Kramer et al. 2004). Another consequence of the development of nisin resistance is an alteration in cell-membrane hydrophobicity, which can be attributed to alterations in fatty acid composition (Martinez and Rodriguez 2005). As noted previously there can be negative consequences associated with the development of resistance; for example, nisin resistance in *L. monocytogenes* results in an acid-sensitive phenotype (van Schaik et al. 1999; McEntire et al. 2004).

Molecular analysis of the response of cells upon exposure to nisin has highlighted the complex nature of the associated reaction. More specifically, Kramer et al. (2006) undertook a study to investigate the impact that exposure to nisin has on the transcriptome of *L. lactis* IL1403 and a nisin-resistant variant thereof (75-fold higher nisin resistance). Alterations in the expression of genes with roles in cell-wall biosynthesis, energy metabolism, phospholipids and fatty acid metabolism, regulatory functions, and metal and/or peptide transport and binding were apparent in the mutant. While a number of these genes have previously been associated with the general stress response of the strain, the involvement of several specific operons was thought to be significant such as the *dlt* operon, involved in the synthesis of D-alanyl residues for the decoration of LTA (lipoteichoic acid), the *arc* operon, encoding genes involved in the arginine deiminase pathway, the *gal* operon, involved in galactose metabolism as well as putative arsenic resistance and bacitracin resistance operons (Kramer et al. 2006; Fig. 5.4). Involvement of the *gal* and *dlt* operons in the nisin resistance of *S. bovis* has previously been reported (Mantovani and Russell 2001) the *dlt* operon also contributes to the innate lantibiotic resistance of *S. aureus* (Peschel et al. 1999) and *L. monocytogenes* (Abachin et al. 2002). Other *L. lactis* genes that are upregulated in the nisin-resistant mutant include those encoding penicillin-binding protein (pbp2A) and the *yedEF* genes. The involvement of a PBP is consistent with previous observations (Gravesen et al. 2001; Kramer et al. 2006). In addition the altered expression of *yedEF* is noteworthy as these encode a phosphotransferase and such systems have also been linked to class II bacteriocin resistance



**Figure 5.4.** Putative nisin resistance mechanisms in *L. lactis*. Four mechanisms for nisin resistance in *L. lactis* have been proposed by Kramer et al. (2006) based on transcriptome analysis in *L. lactis*. Mechanism 1, increasing membrane fluidity may prevent insertion of nisin into the membrane; mechanism 2, cell wall thickening could prevent nisin reaching lipid II in the cytoplasmic membrane. Alterations in the cell wall charge or other factors could affect interactions with nisin; mechanism 3, increasing the pH at the cell wall may result in the degradation of nisin or may force it to bind at the cell wall; mechanism 4, transporters might be utilized to transport nisin from the cell membrane, preventing it from reaching lipid II. Adapted from Kramer et al. (2006).

(Gravesen et al. 2002b; Ramnath et al. 2004; Xue et al. 2005). An insertional mutagenesis approach has also been taken to generate lactacin 3147-resistant mutants of *L. lactis*. The genes, which when interrupted resulted in enhanced lantibiotic resistance, were *mleS*, involved in malolactate fermentation, *yjjC*, encoding an ABC transporter, *ymcF*, encoding a peptidoglycan bound protein, *pi321*, encoding a phage protein, and *tra9811*, encoding a transposase (Guinane et al. 2007). Even though the emergence of lantibiotic resistance is in general a worrisome prospect, in this instance the targeted creation of nisin-resistant lactococci could be beneficial, particularly in the case of strains that serve as starters/adjuncts in foods containing nisin. The mutation of a number of additional genes in a variety of pathogenic species has also been shown to result in an altered response to nisin. Like the *dlt* genes, *mprF* impacts on the charge of the cell envelope and thus also contributes to innate bacteriocin resistance in a number of strains. MprF is responsible for lysinyl-

ation of membrane phospholipids and the increased positive charge of the cell membrane resulting from its enzymatic activity protects pathogens (*L. monocytogenes* and *S. aureus*) from cationic antimicrobial peptides and lantibiotics (Peschel et al. 2001; Thedieck et al. 2006). The manipulation of gene regulators can also impact on nisin sensitivity. Mutation of the gene encoding the general stress response sigma factor  $\sigma^B$  in *L. monocytogenes* results in nisin sensitivity (Begley et al. 2006) whereas deletion of the gene encoding LisK (the histidine kinase component of the two component system, LisRK) results in enhanced resistance to nisin (Cotter et al. 2002).

Resistance to class II bacteriocins is a relatively frequent occurrence (Dykes and Hastings 1998; Limonet et al. 2002) with cell-surface alterations and alterations in fatty acid ratios that result in altered cell membranes again being a common theme (Limonet et al. 2002; Sakayori et al. 2003). Resistance to class II bacteriocins has most

frequently been investigated with respect to factors that impact on what is now known to be the class IIa receptor. One of the first genes linked to class IIa resistance was *rpoN*, in that mutagenesis of the  $\sigma^{54}$  subunit of RNA polymerase that it encodes resulted in a resistant phenotype (Robichon et al. 1997; Dalet et al. 2000). It was also established that a *Listeria* mutant that lacked a mannose-specific PTS enzyme component was resistant to both leucocin A and pediocin PA-1 (Ramnath et al. 2000). A link between the two genes was made when it was established that  $\sigma^{54}$  regulates expression of mannose PTS permease genes (Dalet et al. 2001; Hechard et al. 2001). A number of other researchers linked the mannose PTS and the activity of class IIa bacteriocins (Gravesen et al. 2002b; Xue et al. 2005) and it was noted that it was possible to make a previously bacteriocin-resistant species sensitive to class IIa bacteriocins by expressing the *mptACD* (mannose PTS) operon (Ramnath et al. 2004). As noted above, Diep et al. (2007) ultimately confirmed that the mannose PTS is used as a receptor for class IIa bacteriocins with the man-PTS components IIC and IID being primarily involved in sensitivity.

Nisin has a 50-year track record of safe usage in food industry where resistance development has not been problematic. However, this does not guarantee that resistance will not become an issue in the future, especially if bacteriocins are applied in clinical settings. For this reason there has been an increased focus on researching the basis for bacteriocin resistance in recent years; however, despite this there remains much that has yet to be discovered. Further insights into resistance development can add to our understanding of mode of action, can yield useful markers to monitor potential resistance development and, most importantly, allow us to develop strategies to minimize the likelihood of such an occurrence.

## 5.5. Conclusions

While the great potential of bacteriocins and especially bacteriocins of the LAB has been highlighted previously, recent developments have made the real-

ization of this potential a more likely prospect than ever before. LAB bacteriocins are especially promising because for many species and strains there is a history of safe and effective use. In fact humankind has inadvertently benefited from the protective impact of bacteriocin production by LAB in fermented foods for millennia. In the last century developments were made that made it possible to identify, purify, and structurally characterize bacteriocins. These approaches have been further improved upon in recent years, resulting in a variety of new developments that have greatly enhanced our understanding of mode of action, structure, and production of these compounds.

To date, only nisin and pediocin PA-1 have been applied commercially in food applications where they are used to protect against spoilage and pathogenic organisms. However, other bacteriocins could be at least as effective for food processors as it is possible to apply them with hurdle approaches to enhance food safety and quality, particularly in light of consumer demands for minimally processed, safe, preservative-free foods. The application of bacteriocins could also be further extended to the area of uncooked ready-to-eat vegetables where a bacteriocin wash could be an inexpensive solution. As noted above, the potent activity of many bacteriocins against Gram (+) bacteria has led to their investigation as antimicrobials for clinical application. Given that many of the most notorious drug-resistant pathogens (including MRSA, VRE, and *C. difficile*) are Gram (+) the vast potential of bacteriocins is clear. It is already apparent that lantibiotics such as nisin and lactacin 3147 are capable of killing these notorious pathogens and it is thus evident that these bacteriocins or derivatives therefore could be added to the arsenal of weapons currently used to fight infection. In particular, there is sufficient evidence available to suggest that bioengineering of these peptides will yield even more useful compounds with improved properties for applications in health and industry. Therefore, it would seem that the knowledge that has been accrued about these peptides in recent decades will finally be applied to allow these peptides to reach their full potential.



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## Chapter 6

# Bacteriophages of Lactic Acid Bacteria

Ana Rodríguez González, Pilar García, and Raúl R. Raya

*Due to their important economical impact on faulty food fermentation processes, many virulent and temperate bacteriophages of lactic acid bacteria (LAB) have been thoroughly characterized. To date, 47 complete genome sequences are available in databases. Five natural phage resistance mechanisms in many LAB strains have been described: adsorption inhibition, blocking of DNA penetration, restriction modification systems, acquired phage resistance mediated by clustered regularly interspaced short palindromic repeats systems, and abortive infection. Molecular genetics has been applied in the analysis of phage-host relationship and in strain improvement programs. An overview of the biology, morphology, genomic organization, and molecular analysis of the temperate bacteriophage A2, one of the best Lactobacillus characterized phage, is presented.*

### 6.1. Introduction

Lactic acid bacteria (LAB) have been used for centuries in the preservation and production of fermented foods of plant and animal origins. One of the most critical problems in these processes is the contamination of the starters by bacteriophages (phages) that cause bacterial lysis, and significant economic losses.

The significance of phage infections in dairy fermentations was first highlighted by Whitehead and Cox (1935). Since then, the scientific community has focused its efforts in controlling phage contamination to develop strategies for their elimination

from the dairy environment. These strategies have been based on strain selection and rotation systems, phage-insensitive mutants, and antiphage resistance mechanisms. Nevertheless, phage remains the major single cause of fermentation failure. *Lactococcus lactis* and *Streptococcus thermophilus* strains suffer the highest number of phage infections. Consequently, the present knowledge on phage biology mainly relies on phages infecting these species. Phages infecting lactobacilli species have been less studied because they hardly cause problems in dairy processes. However, the increasing role of lactobacilli as probiotics has fuelled the research into their phages. In fact, quite a number of dairy fermentation batches in which *Lactobacillus casei*, *Lactobacillus acidophilus*, *Lactobacillus delbrueckii* subsp. *Lactis*, and *Lactobacillus helveticus* strains have suffered phage contamination have been reported.

The study of LAB phages has not only brought information about the origin of lytic phages in the fermentation industry but, in particular, streptococcal comparative phage genomics has also significantly contributed to the field of phage taxonomy and to the characterization of the clustered regularly interspaced short palindromic repeats (CRISPR) system, an antiphage mechanism acquired by bacteria after phage infection. Some LAB phages have been used as transducing vehicles for genetic characterization as well as LAB strain manipulation. Furthermore, phage genetic elements have been the DNA source in developing antiphage systems (i.e., using phage promoter sequences or repressor function) and integration vectors (i.e., using integrative

functions) to deliver and stabilize genes in the LAB genome. Excellent reviews on the field of LAB phages have been published recently (Josephsen and Neve 2004; Brøndsted and Hammer 2006; Brüssow 2006; Brüssow and Desiere 2006; Brüssow and Suárez 2006; Emond and Moineau 2007).

## 6.2. Bacteriophages of LAB

Phages can be divided into two classes based on lifestyle: virulent or temperate. Virulent phages can only multiply by means of a lytic cycle; the phage replicates its genome and assembles hundreds of new progeny, which is then released after cell lysis. Temperate phages, in contrast, have a choice of reproductive modes when they infect a new host cell. Sometimes the infecting phage initiates a lytic cycle, resulting in lysis of the cell and release of new phages, as previously shown. The infecting phage may alternatively initiate a lysogenic cycle instead of replicating the phage genome assuming a quiescent state called prophage, often integrated into the host genome but sometimes maintained as a plasmid. Bacteria carrying a prophage are said to be lysogenized or lysogenic (capable of producing lysis) since, under certain conditions, the prophage is induced into the lytic cycle of phage replication. The transition from lysogeny to lytic development, called lysogenic or prophage induction, occurs in response to DNA damage (i.e., after treatment with mitomycin C). The lytic infection process is very much dependent on the host metabolic machinery, so in most cases it is highly affected by what the host was experiencing shortly prior infection such as the energetic state, nutrients, and environmental conditions present during the infection process itself (Guttman et al. 2005). The frequency of lysogenization also depends on the growth conditions and on the multiplicity of infection; starvation of cells prior to phage adsorption and high multiplicity of infection enhance lysogeny. On the contrary, when the cell energy level is high, phage favors its lytic cycle. As discussed by Levin and Lenski (1985), the lysogenic state is highly evolved, requiring coevolution of phage and host that presumably reflects various advantages to both. Temperate phages can

help protect their host from infection by other phages and can lead to significant changes in the properties of their hosts, including lysogenic conversion, restriction systems, and resistance to antibiotics and other environmental insults.

LAB phages have been isolated from diverse natural sources (e.g., raw milk, yogurt, cheese, sauerkraut, pickles, cereals, wine, coffee, sewage, saliva, human vagina) and upon induction of lysogens. Most of the isolated phages showed very restricted host ranges, limited to one or a few starter strains of the same species; for most lysogens, suitable indicator strains or conditions for lytic propagation are not easily defined and evidence of these phage-like particles is limited to their visualization under the transmission electron microscope. The narrow host range that most temperate phages exhibit suggests the presence of homoimmune or restriction modification systems. Plasmid DNA transduction has been used as an alternative to lytic plaque assays to define the host range of a *Lactobacillus* phage, phi-adh; high-frequency transducing particles of phi-adh transduced plasmid DNA to several *Lactobacillus gasseri* cells that, otherwise, did not support lytic growth of this phage (Raya et al. 1989). The presence of prophage sequences can also be pointed in the completed genome sequence of LAB strains by using markers as phage integrase, portal protein, terminase, and tail tape measure protein (Canchaya et al. 2003). Comparative analysis of complete bacterial genomes has shown that most LAB prophage genomes share the same genetic organization, involving two clusters of genes that control related functions, which are transcribed divergently. One of these clusters comprises genes participating in integration and lysogeny maintenance; the other includes genes involved in lytic development (Canchaya et al. 2003, 2004). Also, prophage sequences are located at both sides of the terminus of the bacterial replication, the lytic gene cluster being transcribed in the same direction of the surrounding bacterial genes (Canchaya et al. 2004).

LAB phages are members of the Caudovirales order that comprises over 95% of known phages (Forde and Fitzgerald 1999). These phages possess



double-stranded linear DNA and their G+C content is similar to that of their hosts, ranging from 33% in *Lact. delbrueckii* subsp. *lactis* phages to 48% in *Lact. casei* phages. The genome size ranges from 14 to 141 kb (Table 6.1). Most lactococcal phages

belong to the Siphoviridae family (long, non-contractile tails) while a few belong to the Podoviridae family (short tails). A recent classification of lactococcal phages proposed eight groups instead of the former 12 groups (Deveau et al. 2006). Lysogeny is

**Table 6.1.** LAB phage genomes available in database.

Phage	Genome Size (bp)	GC (%)	ORFs		tRNA RefSeq	Reference
Siphoviridae						
<i>Lactobacillus</i>						
A2(T)	43411	44.9	62	—	NC_004112	Alvarez et al. 1998
LL-h (V)	34659	47.8	51	—	NC_009554	Mikkonen et al. 1994
Lc-Un (V)	36466	44.3	51	—	NC_007501	Tuohimaa et al. 2006
phiAT3 (T)	39166	44.6	55	—	NC_005893	Lo et al. 2005
Lrm1 (T)	39989	45.5	54	—	NC_011104	Durmaz et al. 2008
phi-g1e (T)	42259	43.1	62	12	NC_004305	Kodaira et al. 1997
Lj928 (T)	38384	34.7	50	1	NC_005354	Ventura et al. 2004
Lj965 (T)	40190	35.1	46	4	NC_005355	Ventura et al. 2004
phiadh(T)	43785	35.6	65	—	NC_000896	Altermann et al. 1999
Lv-1	38934	37.0	47	—	NC_011801	Unpublished
KC5a	38239	36.9	61	—	NC_007924	Unpublished
<i>L. lactis</i>						
1706 (V)	55597	33.7	76	—	NC_010576	Garneau et al. 2008
4268 (V)	36596	35.4	49	—	NC_004746	Trotter et al. 2006
Jj50 (V)	30510	33.9	55	—	NC_008370	Mahony et al. 2006
712 (V)	28538	34.7	58	—	NC_008363	Mahony et al. 2006
P008 (V)	27453	34.9	49	—	NC_008371	Mahony et al. 2006
Q54 (V)	26537	37.1	47	—	NC_008364	Fortier et al. 2006
IL170 (V)	31754	34.3	64	—	NC_001909	Crutz-Le Coq et al. 2002
sk1 (V)	28451	34.5	56	—	NC_001835	Chandry et al. 1997
BK5-T (T)	40003	35.0	63	—	NC_002796	Desiere et al. 2001
P087 (V)	60074	34.4	88	5	NC_012663	Villion et al. 2009
TP901-1 (T)	37667	35.4	56	—	NC_002747	Brøndsted et al. 2001
Tuc2009 (T)	38347	36.2	56	—	NC_002703	Arendt et al. 1994
(V)	29305	34.7	54	—	NC_011046	Szczepańska et al. 2007
bIL67 (V)	22195	36.0	37	—	NC_001629	Schouler et al. 1994
c2 (V)	22172	36.3	39	2	NC_001706	Lubbers et al. 1995
LC3 (T)	32172	35.5	51	—	NC_005822	Blatny et al. 2004
r1t (T)	33350	35.5	50	—	NC_004302	van Sinderen et al. 1996
ul36	36798	35.8	61	—	NC_004066	Labrie and Moineau 2002
bIL285	35538	35.2	62	—	NC_002666	Chopin et al. 2001
bIL286	41834	35.3	61	—	NC_002667	Chopin et al. 2001
bIL309	36949	35.7	56	—	NC_002668	Chopin et al. 2001
bIL310	14957	35.9	29	—	NC_002669	Chopin et al. 2001
bIL311	14510	34.2	22	—	NC_002670	Chopin et al. 2001
bIL312	15179	33.0	27	—	NC_002671	Chopin et al. 2001
<i>Strep. thermophilus</i>						
Sfi11 (V)	39807	38.6	53	—	NC_002214	Lucchini et al. 1999
Sfi19 (V)	37370	38.3	45	—	NC_000871	Desiere et al. 1998
Sf21 (T)	40739	37.6	50	—	NC_000872	Desiere et al. 1998

Table 6.1. Continued

Phage	Genome Size (bp)	GC (%)	ORFs		tRNA RefSeq	Reference					
O1205 (T)	43075	38.3	57	—	NC_004303	Stanley et al. 1997					
DT1 (V)	34815	39.1	45	—	NC_002072	Tremblay and Moineau 1999					
858 (V)	35543	39.8	46	—	NC_010353	Deveau et al. 2008					
7201 (V)	35466	38.7	46	—	NC_002185	Le Marrec et al. 1997					
2972 (V)	34704	40.2	44	—	NC_007019	Lévesque et al. 2005					
Podoviridae											
<i>L. lactis</i>											
KSY1 (V)	79232	35.1	130	3	NC_009817	Chopin et al. 2007					
C3 morphotype	18762	33.7	28	—	NC_010363	Kotsonis et al. 2008					
ascphi28											
V, C2 morphotype											
Myoviridae											
<i>Lactobacillus</i>											
LP65 (T)	131522	37.3	165	14	NC_006565	Chibani-Chennoufi et al. 2004					
Lb338-1	141111	—	199	—	NC_012530	Unpublished					

a common phenomenon in *L. lactis* strains (Brøndsted and Hammer 2006).

All *Strep. thermophilus* phages belong to the Siphoviridae family. Lysogeny in this species is rare (less than 10%). Virulent and temperate streptococcal phages are genetically related and form two distinct siphovirus groups according to the number of major structural proteins and the packaging mechanism of their double-stranded DNA: cos-like (group Sfi21) and pac-like (group Sfi11). Sfi11-like phages differ from the Sfi-11 phages by having two distinct major head proteins instead of only one, by using a scaffold protein to aid assembly, and by not processing their major head proteins (Proux et al. 2002).

Regarding lactobacilli phages, the knowledge is much limited and only a few have been deeply studied. These phages are mainly Siphovirus and a few Myoviruses. Lysogeny is widespread throughout the family Lactobacillaceae. For example, *Lact. casei* S-1 strain (ATCC 27139) harbors the temperate phage FSW, which was not inducible with mitomycin C or ultraviolet light, although spontaneous induction was observed (Shimizu-Kadota et al. 1983). Virulent forms that originated from FSW were isolated from abnormal fermentations with S-1

strain. DNA-DNA hybridization and heteroduplex analyses showed that DNA of FSW and its virulent derivatives were identical, except that an extra 1.3 kb piece of DNA was present in the virulent forms. Sequence analysis of this extra fragment (1256 bp) showed characteristics of an insertion sequence (IS) element named ISL1, the first element described in *Lactobacillus* (Shimizu-Kadota et al. 1985). The demonstration that prophage FSW was the source of virulent phages represented the first formal evidence that temperate bacteriophages can be a source for the appearance of virulent variants in dairy fermentations. The close relationship observed between temperate and virulent phages of *Lact. delbrueckii* subsp. *bulgaricus* and *Lact. helveticus* suggested that temperate phages could also be the source of phage outbreaks found in commercial fermentations, although it has not yet been proven unequivocally.

At present, genome sequences of 26 phages (24 Siphovirus and 2 Podovirus) infecting *L. lactis* strains, 8 (Siphovirus) infecting *Strep. thermophilus* strains, and 13 (11 Siphovirus and 2 Myovirus) infecting lactobacilli strains are available in databases (Table 6.1). Likewise, many prophage genome sequences are also available in the sequenced

**Table 6.2.** Strategies applied in phage control.

<b>Survey</b> the reservoir and distribution of phages in the dairy environment, milk, and starters.
<b>Control phage proliferation:</b> includes, among others, direct inoculation of milk with concentrated starter cultures; use of antiphage starter medium; use of separate rooms for starter production and cheese manufacture; regular cleaning and disinfections (chlorinate) of vats, equipment, rooms (laboratory and factory), clothes, and air (positive filtration); rotation of starter strains.
<b>Strain selection and starter rotation system:</b> use of mixture of well-characterized strains; starter rotation system with nonoverlapping phage-insensitive patterns.
<b>Bacteriophage-insensitive bacterial mutants</b>
<b>Design of phage-resistant starter strains:</b> conjugation of natural phage resistance systems (i.e., restriction/modification systems).
<b>Antiphage systems:</b> use of genetic phage elements that interfere with the phage replication cycle, including phage repressor, superinfection exclusion proteins; origin of replication; and anti-messenger of phage genes.

genomes for *L. lactis* and for several species of *Lactobacillus* (Shimizu-Kadota et al. 2000; García et al. 2003; Lo et al. 2005).

### 6.3. Bacteriophage Resistance Systems

Table 6.2 summarizes the phage control strategies applied at the dairy industry. Selection of appropriate combinations of bacterial starters with different phage sensitivity and an efficient starter rotation system are the most used tools to cope with industrial phage problems.

Molecular genetics has been applied for the analysis of phage-host relationship and contributed to the discovery of natural resistance mechanisms in many LAB strains, which have been exploiting in strain improvement programs (Sturino and Klaenhammer 2006). Natural phage resistance mechanisms have been mainly studied in lactococci, and more recently in *Strep. thermophilus*, and categorized according to the step of action in the lytic cycle (Josephsen and Neve 2004). Five mechanisms were well described: adsorption inhibition, blocking of DNA penetration, restriction modification systems, acquired phage resistance mediated by CRISPR systems, and abortive infection. Genes encoding 22 phage abortive infection systems (Abi), 29 restriction modification (R/M) systems, 8 adsorption inhibition (Ads), and 2 injection blocking have been identified (Emond and Moineau 2007).

Prevention of adsorption is an effective means of phage resistance. A masking or lack of the appropri-

ate phage receptors in the host cell wall occurs (Forde and Fitzgerald 1999). Likewise, phage DNA injection can be prevented by mutations of gene encoding the phage infection protein (Pip) (Garbutt et al. 1997) or by the presence in the bacterial genome of gene *sie*<sub>2009</sub> involved in a superinfection-exclusion mechanism (McGrath et al. 2002). *Sie*-specifying genes are prophage-encoded but dissemination differences have been observed between *L. lactis* MG1363 and IL1403 prophage-encoded *Sie* systems (Mahony et al. 2008).

Abi systems allow phage adsorption and DNA injection but phage development is further interrupted. The death of infected cells occurs but no viral progeny is released. Most Abi systems have been found in *L. lactis*. Some of them (A, F, K, P, T) interfere with DNA replication, or RNA transcription (B, G, U), or causes a limited major capsid protein production (C) or a premature cell lysis (Z; Chopin et al. 2007). Recently, a novel Abi system (V) has been identified preventing cleavage of the replicated DNA (Haaber et al. 2008).

R/M systems reduce the efficiency of plaquing (Moineau 1999). Three types of R/M systems have been identified in LAB, most of them belonging to Type II (LlaDCHI, LlaAI, ScrFI, LlaBI). They involve two distinct enzymatic activities; a host-encoded, site-specific restriction endonuclease digests the phage DNA while cell DNA is modified by the action of a methylase to remain protected.

Most of natural phage defense mechanisms are plasmid-encoded. As these plasmids can be

conjugally transferred, food-grade transconjugant strains have been obtained that show a reliable performance in industrial environment. Overall, a highly effective antiphage system should provide very low efficiency of plating (in the range of  $10^{-7}$  to  $10^{-9}$ ; Coffey and Ross 2002).

*Lact. casei* temperate bacteriophage A2 has also been used as a tool in the generation of bacteriophage resistance systems. A 258-bp region identified as putative replication origin of phage A2 can direct a partial resistance against phage infection (Moscoso and Suarez 2000). This cis-acting genetic element was obtained by cloning ori region in pG<sup>+</sup>host9 allowing the titration of phage-specific replication factors. However, a more efficient strategy was applied to give resistance against phage A2 attack through expression of the phage A2 repressor (Alvarez et al. 1999). Stability of phenotype resistance during milk fermentation was due to the integration of gene CI into the bacteria genome.

### 6.3.1. CRISPR System

Recently it was shown that bacteria acquire resistance to phage attack by incorporation of short nucleotide (spacer) sequences into regions named CRISPR. CRISPR loci contain stretches of short (21–47 bp) direct palindromic repeats, separated by unrelated spacer sequences of similar size. The number of direct repeats and spacers varies from 2 to 200, and changes in response to phage infection by acquiring new spacer sequences from the infecting phage; these changes have been correlated with phage resistance. In addition, CRISPR loci usually contain a 3'-adjacent non-repeat leader region and several CRISPR-associated sequences (CAS) genes (Deveau et al. 2008; Fig. 6.1 (see in the color plate section)).

CRISPR regions are found in about 40% of known eubacterial genomes and most archaeal genomes and show a high level of polymorphism in different strains. In LAB, eight families of CRISPR loci have been identified based on Cas 1 gene (COG 1518; Makarova et al. 2002) and CRISPR repeat

sequences, content, and organization (Barrangou et al. 2007). All these families were also identified in similar loci of microbial species of two different phyla: Firmicutes (Gram [+] low GC% bacteria) and Actinobacteria (Gram [+] high GC% bacteria), which suggest that the diversity of CRISPR in LAB could reflect horizontal gene transfer. Out of 102 LAB genomes analyzed (Horvath et al. 2009), CRISPR loci were found in 8 out of 13 *Lactobacillus*, 10/13 *Streptococcus*, 5/7 *Enterococcus*, 4/6 *Bifidobacterium*, 1/1 *Atopobium*, and 1/1 *Symbiobacterium* genomes. However, CRISPR loci were absent in the genomes of *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Leuconostoc*, and *Carnobacterium* (Deveau et al. 2008). Some LAB genomes contained more than one CRISPR locus (i.e., three distinct CRISPR loci were found in *Strep. thermophilus*; Deveau et al. 2008). The total length of the CRISPR loci in LAB varied between 628 and 3400 bp (Bolotin et al. 2005) with an average of 20 repeats (20–27 long) and spacers (26–40 long).

CRISPRs are RNA elements that protect the cell against foreign genetic elements. Two of the CAS gene products showed characteristic motifs for helicases (superfamily 2) and for exonucleases (RecB family). Transcription of CRISPR regions and processing of transcripts have been experimentally demonstrated in *Escherichia coli* (Brouns et al. 2008). Although the mechanisms of CRISPR function remain to be determined, it was found that these noncoding elements are transcribed to RNA, which are then processed in each direct repeat by the associated enzyme complex named CAS. This complex, assisted by the CAS helicase function, retains the phage-derive spacer sequence that serves as small guide RNA to interfere with phage replication or function. The CAS enzyme complex also incorporates new fragment of phage sequences as new spacers; most (70%) of the spacer sequences were found to be unique, homologous predominantly with one of the phage strands. CRISPR spacer sequences reflect previous mobile DNA infection elements. Thus, the CRISPR system exerts a high selective pressure on phage genomes.

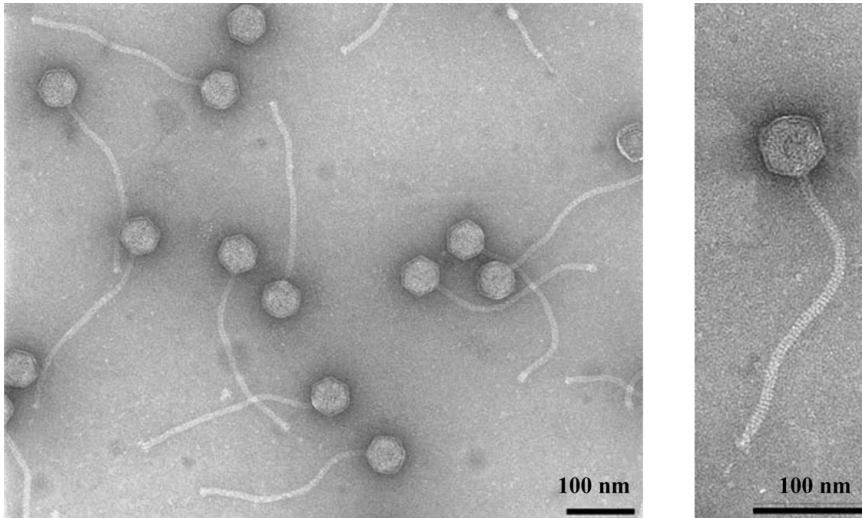
#### 6.4. A Case Study: Bacteriophage A2

Phage A2 is a temperate bacteriophage that infects strains of *Lact. casei* and *Lactobacillus paracasei*. It was isolated from the whey of Gamonedo cheese, a long-ripening homemade blue cheese manufactured in Asturias, Northern Spain (Herrero et al. 1994). A2 belongs to the family Siphoviridae being composed by an isometric head (60 nm in diameter) and a non-contractile tail (280 × 12 nm) ended by a basal plate with a protruding spike (28 nm; Fig. 6.2). One-step growth kinetics of lytic development revealed a latent period of 140 min and a burst size of about 200 PFU/infected cell (Herrero et al. 1994). Its genome consists of a double-stranded DNA molecule 43,411 kb long with 3'-protruding cohesive ends. It encodes 61 open reading frames (ORFs) grouped in packaging, morphogenesis, host lysis, integration, lysis-lysogeny switch, and DNA replication modules (see Fig. 6.3 in the color plate section). Out of the 61 orf, 55 are transcribed in the same direction while orf20 to 23 are transcribed in the opposite direction. A putative transcriptional terminator and the phage attachment site (*attP*) were identified at the intergenic region between orf19 and

20. Orf23 and 24 encode CI and cro proteins, respectively (García et al. 2003).

The *packaging module* consists of orf61, orf1, and orf2 genes. Orf61 gene product is the small subunit of the terminase enzyme (16.5 kDa) while the product encoded by orf2 (64.2 kDa) showed homology with the putative large terminase subunits of other phages (García et al. 2003). Both genes are separated by the cohesive ends (13 nt-long 3'-protruding single stranded ends of complementary base sequence).

The *morphogenesis module* consisted of 12 ORFs. The products of orf3 and orf4 are a portal protein (Gp3; 46.1 kDa) and a phage maturation protease (Gp4; 23.6 kDa), respectively. The major head protein gene, orf5, encodes two polypeptides of different sizes: Gp5A (35 kDa) resulting from canonical translation and further proteolytic processing, and Gp5B (42 kDa; 85 amino acids) generated by a 1 ribosomal frameshift at the penultimate codon of orf5 mRNA. A CCCAAAA slippery region and a stem-loop that begins 9 nt after the end of the slippery sequence are the two cis-acting elements needed for the above frameshift. Both proteins share their amino termini and are the major components



**Figure 6.2.** Electron micrograph of negatively stained bacteriophage A2 that infects strains of *Lact. casei* and *Lact. paracasei*.



of the virion head (ratio 4:1). They are essential for the generation of viable phages since lysogens harboring prophages that produce only one of these proteins did not show viable progeny after mitomycin C induction (García et al. 2004). Orf6 encodes a putative DNA packaging protein while orf7 to orf9 encode head-tail joining proteins. The major tail protein gene, orf10, uses a -1 translational frameshift promoted by a similar slippery sequence and an RNA pseudoknot located at 3' of the gene to generate two polypeptides (21.8 kDa and 29.6 kDa). Orf11 has not a predicted function and orf12 codes for a predicted tape measure protein. At the end of the morphogenesis module, orf13 and 14 encode for a hypothetical protein and a host interacting protein, respectively.

The *cell lysis module* is composed of two genes, orf17 and orf18, that encode for holin (14.8 kDa) and lysin (37.4 kDa), respectively, two proteins involved in bacterial cell degradation. On the other hand, orf19 could be a gene related to lysogenic conversion, although this function has yet not been proven.

The *integration module* includes orf20 encoding the site-specific integrase (43.8 kDa) that mediates recombination between the phage- (*attP*) and bacterial- (*attB*) attachment sites and phage integration at the end of a host tRNA<sup>Leu</sup> gene. Orf21 could encode the phage excisionase. Both functions have already been tested (Alvarez et al. 1998, 1999).

The *genetic switch module* directs phage development into the lytic or lysogenic cycle. It has a unique operator region (Op) with two divergently transcribed promoters (P<sub>L</sub> and P<sub>R</sub>). P<sub>L</sub> promotes transcription of orf23 that encodes the CI-like repressor (25.2 kDa), and P<sub>R</sub> promotes transcription of orf24 that encodes Cro repressor (9.1 kDa). These two genes are separated by a short intergenic region in which three 20-bp inverted repeat operator sequences (O<sub>1</sub>, O<sub>2</sub>, O<sub>3</sub>) are identified. Different affinity of the two repressors for the different operators was observed. CI binds preferentially to the O<sub>1</sub> and O<sub>2</sub> subsites, shutting off transcription from P<sub>R</sub>, while enhancing expression from P<sub>L</sub> so that lysogeny becomes established (García et al. 1999). Conversely, Cro binds preferentially to the O<sub>3</sub> subsite. This

results in repression of transcription from P<sub>L</sub> and abolition of CI production, and consequently, phage enters the lytic cycle (García et al. 1999; Ladero et al. 1999). It is worth noticing that *Lact. casei* derivatives containing a chromosomal copy of CI are completely resistant to phage infection (Alvarez et al. 1999).

Downstream of the *lysis/lysogeny module* is located an 8 kb DNA fragment involved in DNA replication in which 15 ORFs were identified (Moscoso and Suarez 2000). Notably, orf33 encodes a 50 kDa protein that shows similarity with DNA helicases; orf24 encodes a single-stranded binding protein (19.8 kDa) and orf35 shows similarity to phage DNA primases. The *origin* sequence is located downstream of orf35. The region from orf40 to orf49 seems to be dispensable as viable mutants showing 1 to 3 kb deletions were obtained (García et al. 2003). A putative DNA methylase (orf47) and an endonuclease (orf48) are contained within the dispensable region. Orf57 also encodes an endonuclease.

#### 6.4.1. Comparative Analysis

As of February 2009, phage genomic studies have provided the complete genomes of 526 phages (<http://www.ncbi.nlm.nih.gov/genomes/>). Out of these, 25 are lactococcal phages; 14 are lactobacilli phages; 8 are *Strep. thermophilus* phages; and 1 is a *Leuconostoc* phage.

Genome similarity is shown by phages isolated from closely related species such as *L. gasseri* (Lga1) and *Lactococcus johnsonii* (Lj775). It is suggested that recombination events could favor exchange of individual genes, gene fragments, or even functional modules (Ventura et al. 2006).

A previous comparative genome analysis (Proux et al. 2002) revealed that phage A2 shows an identical organization of replication and lysogeny modules to *Strep. thermophilus* phage Sf21. The Sf21-like genus includes, besides phage A2, *Strep. thermophilus* phage 7201 and different prophages harbored by *L. lactis* strain IL1403. In addition, a similar genomic organization of phage A2 is shown by *Lact. casei* temperate phages phi-AT3 and LcaI, *Lactobacillus*

*rhamnosus* virulent phage Lc-Nu, and prophage Lmr1. Particularly, packaging and morphogenetic clusters and CI and Cro proteins show high similarity with genomic phage sequences of Lmr1 (Durmaz et al. 2008). Similarity was also observed with streptococcal phages for the DNA replication genes

(Moscoso and Suarez 2000). Bioinformatic analysis of phage A2 protein sequences showed that five proteins shared higher conserved amino acids sequence with several Gram (+) infecting bacteriophages (Table 6.3). The terminase large subunit and tail tape measure protein included in the packaging/

**Table 6.3.** Highest score of bacteriophage A2 proteins in bacteriophage databases.

A2 protein	Phage (score >200)	Identity (%)/ similarity (%)	E value	Accession number
Terminase large subunit (gp2)	<i>Lactobacillus</i> phage Lrm1	95/98	0	reflYP_002117670.1
	<i>Streptococcus</i> phage 9429.1	50/70	2e-160	reflYP_596301.11
	prophage Lp3 <i>Lactobacillus plantarum</i> WCFS1	36/60	2e-99	reflNP_785918.11
	<i>Staphylococcus</i> phage PVL	34/56	3e-94	reflNP_058441.11
	<i>Staphylococcus</i> phage tp310-1	34/56	1e-93	reflYP_001429870.11
	<i>Lactococcus</i> phage bIL309	31/54	8e-74	reflNP_076734.11
	<i>Streptococcus</i> phage Sfi21	30/53	2e-69	reflNP_049967.11
Portal protein (gp3)	<i>Lactobacillus</i> phage Lrm1	92/96	0.0	reflYP_002117672.11
Protease (gp4)	<i>Lactobacillus</i> phage Lrm1	90/95	3e-104	reflYP_002117673.11
Major head protein (gp5)	<i>Lactobacillus</i> phage Lrm1	94/96	0.0	reflYP_002117674.11
	<i>Streptococcus</i> phage 9429.1	44/65	2e-90	reflYP_596306.11
	<i>Lactobacillus</i> phage phiAT3	42/57	5e-68	reflYP_025031.11
	<i>Lactobacillus</i> phage Lc-Nu	40/58	1e-66	reflYP_358764.11
	<i>Lactococcus</i> phage bIL285	33/50	9e-52	reflNP_076616.11
Head-tail joining protein (gp7)	<i>Lactobacillus</i> phage Lrm1	95/96	2e-78	reflYP_002117676.11
Head-tail joining protein (gp8)	<i>Lactobacillus</i> phage Lrm1	99/100	9e-70	reflYP_002117677.11
Head-tail joining protein (gp9)	<i>Lactobacillus</i> phage Lrm1	96/97	2e-54	reflYP_002117678.11
Major tail protein (gp10)	<i>Lactobacillus</i> phage Lrm1	94/96	2e-101	reflYP_002117679.11
Tape measure protein (gp12)	<i>Lactobacillus</i> phage Lrm1	83/90	0.0	reflYP_002117681.11
	<i>Listeria</i> phage A006	33/53	7e-129	reflYP_001468855.11
	<i>Streptococcus</i> phage 700P1	49/70	1e-55	gblABB02692.11
	<i>Staphylococcus</i> phage 3A	30/47	5e-55	reflYP_239947.11
Tail protein (gp13)	<i>Lactobacillus</i> phage Lrm1	58/72	0.0	reflYP_002117682.11
	<i>Lactobacillus</i> phage Lc-Nu	35/50	5e-71	reflYP_358772.11
Host interaction protein (gp14)	<i>Lactobacillus</i> phage Lrm1	77/86	0.0	reflYP_002117683.11
	<i>Lactobacillus</i> phage phiAT3	48/61	0.0	reflYP_025042.11
	<i>Lactobacillus</i> phage Lc-Nu	50/64	1e-138	reflYP_358773.11
	<i>Lactobacillus rhamnosus</i> Lc-Nu-like prophage	49/65	2e-136	gblAAX07959.11
Lysin (gp18)	<i>Lactobacillus</i> bacteriophage PL-1	94/96	0.0	dbjlBAA96749.11
	<i>Lactococcus</i> phage KSY1	53/67	9e-95	reflYP_001469072.11
CI (gp23)	<i>Lactobacillus</i> phage Lrm1	98/99	2e-115	reflYP_002117694.11
	<i>Lactobacillus</i> phage Lc-Nu	85/88	2e-63	reflYP_358780.11

Table 6.3. Continued

A2 protein	Phage (score >200)	Identity (%)/ similarity (%)	E value	Accession number
NTP-binding protein (gp34)	<i>Lactobacillus</i> phage Lc-Nu	92/96	2e-106	reflYP_358787.11
	<i>Lactobacillus</i> phage phiAT3	90/94	9e-103	reflYP_025055.11
	<i>Lactobacillus</i> phage Lrm1	89/94	3e-102	reflYP_002117702.11
	<i>Streptococcus</i> phage Sfi19	59/73	2e-76	reflNP_049951.11
	<i>Streptococcus</i> phage O1205	58/74	3e-76	reflNP_695087.11
	<i>Streptococcus</i> phage Sfi11	58/73	1e-75	reflNP_056708.11
Helicase (gp36)	<i>Lactobacillus</i> phage phiJL-1	54/69	2e-110	reflYP_223915.11
	prophage LambdaSa03 <i>Streptococcus agalactiae</i> A909	47/67	1e-102	reflYP_329249.11
	<i>Lactobacillus</i> phage phiadh	44/62	3e-102	reflNP_050128.11
	<i>Listeria</i> phage 2389	46/64	6e-102	reflNP_511031.11
	<i>Streptococcus</i> phage O1205	41/62	3e-96	reflNP_695088.11
	<i>Streptococcus</i> phage 2972	40/62	6e-96	reflYP_238518.11
	<i>Streptococcus</i> phage Sfi19	41/62	6e-96	reflNP_049952.11
	<i>Streptococcus</i> phage Sfi21	41/62	6e-96	reflNP_049999.11
	<i>Lactococcus</i> bacteriophage phi31	40/62	2e-95	emblCAC04160.11
Primase (gp38)	<i>Listeria</i> phage 2389	49/65	0.0	reflNP_511033.11
	<i>Lactobacillus</i> phage phiadh	49/67	0.0	reflNP_050131.11
	<i>Listeria</i> phage B025	49/64	0.0	reflYP_001468699.1
	<i>Bacillus</i> phage phi105	36/56	4e-136	reflNP_690795.11
Endodeoxyribonuclease (gp51)	<i>Lactobacillus</i> phage Lrm1	60/71	2e-53	reflYP_002117719.11
	<i>Lactobacillus</i> phage phiAT3	61/71	3e-52	reflYP_025078.11
Terminase small subunit (gp64)	<i>Lactobacillus</i> phage Lrm1	98/100	5e-127	reflYP_002117722.11

morphogenetic module and NTP-binding protein, helicase and primase located in the replication module have identity percentages ranging from 95% to 30%. These results confirm the importance of horizontal gene exchange in the bacteriophage evolution and fulfill the reticulate representation of relationships between phage genomes (Lima-Mendez et al. 2008).

## 6.5. Conclusions

Phage DNA is one of the main vectors for lateral gene transfer between bacteria and a major force of bacterial diversity and evolution. It is expected that the characterization of an increasing number of LAB phage genomes should lead to a better understanding of phage evolution required for the development of long-term phage-resistant LAB strains.

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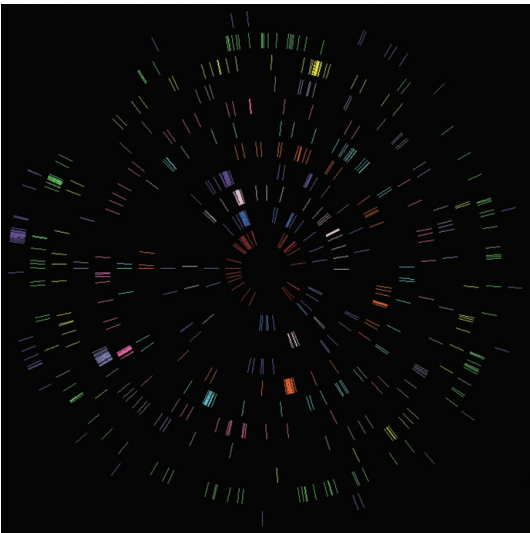
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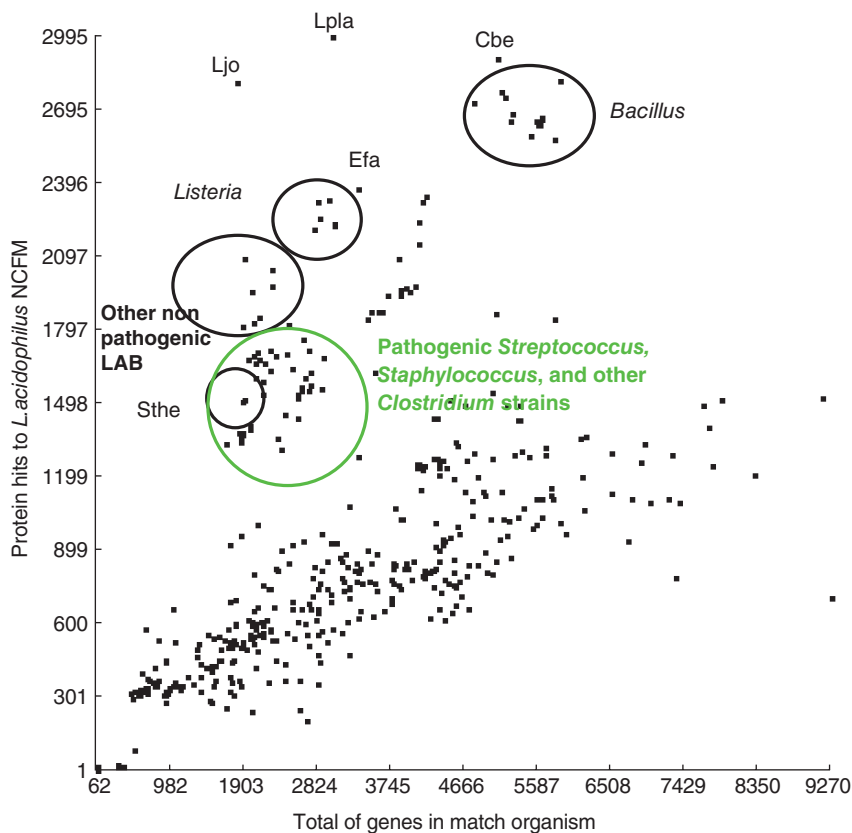




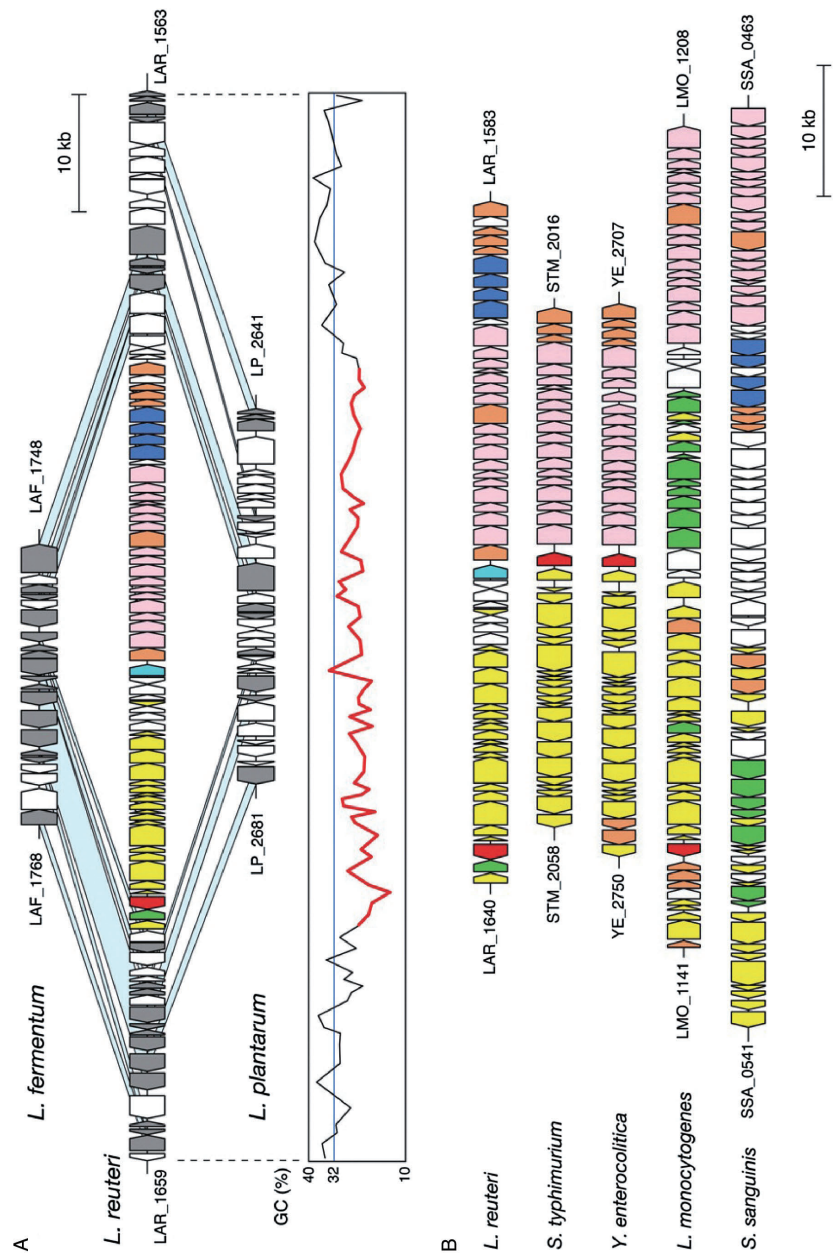
Reference DNA molecule: Chromosome *Clostridium difficile* QCD-32g58

Comparison DNA molecules (chromosome)	Number of protein matches to reference DNA molecule
<i>Enterococcus faecalis</i> V583	137
<i>Lactobacillus plantarum</i> WCFS1	81
<i>Lactobacillus sakei</i> 23K	80
<i>Lactococcus lactis</i> subsp. <i>lactis</i> IL1403	72
<i>Lactobacillus salivarius</i> subsp. <i>salivarius</i> UCC118	72
<i>Lactobacillus brevis</i> ATCC 367	61
<i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i> ATCC BAA-365	55
<i>Lactobacillus johnsonii</i> NCC 533	52
<i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i> ATCC 11842	51
<i>Lactobacillus acidophilus</i> NCFM	50
Total number of reference-comparison protein matches:	711

**Figure 2.2.** Multi-genome homology comparison showing protein-level matches between *Clostridium difficile* QCD-32g58 used as single reference DNA molecule, and multiple LAB species. The cutoff selection for protein matches was 60% minimum percent similarity. The table lists the genomes representing each single circle in outermost-innermost direction. The comparison was performed using the Multi-Genome Homology Comparison tool in the Comprehensive Microbial Resource web site at JCVI (<http://www.jcvi.org/>).

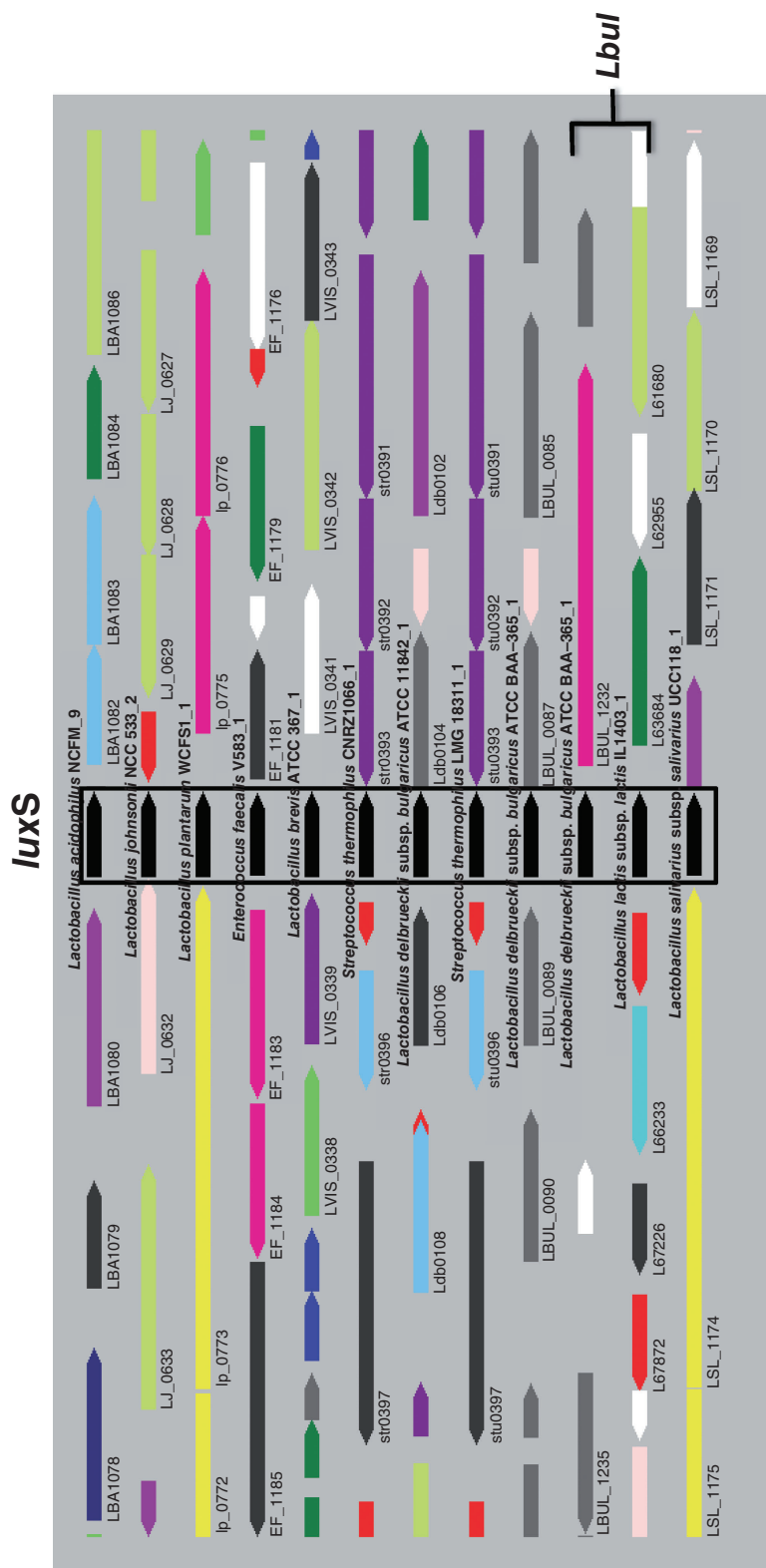


**Figure 2.3.** Genome homology diagram depicting the number of total protein similarities between *Lactobacillus acidophilus* NCFM (reference DNA molecule, x-axis) and all bacterial genome sequences available in the Comprehensive Microbial Resource web site at JCVI (<http://www.jcvi.org/>). The parameters used for the analysis were minimum percent similarity 40%, minimum percent identity 10%, and maximum *P* value 0.0001. Total protein hits (including multiple hits per protein) option was selected. Genomes annotated in the figure are Ljo, *Lactobacillus johnsonii* NCC 533 (Pridmore et al. 2004); Lpl, *Lactobacillus plantarum* WCFS1 (Kleerebezem et al. 2003); Cbe, *Clostridium beijerinckii* NCIMB 8052 (Genebank number CP000721); Efa, *Enterococcus faecalis* V583 (Paulsen et al. 2003); Sthe, *Streptococcus thermophilus* LMG 18311 and CNRZ1066 (Bolotin et al. 2004).

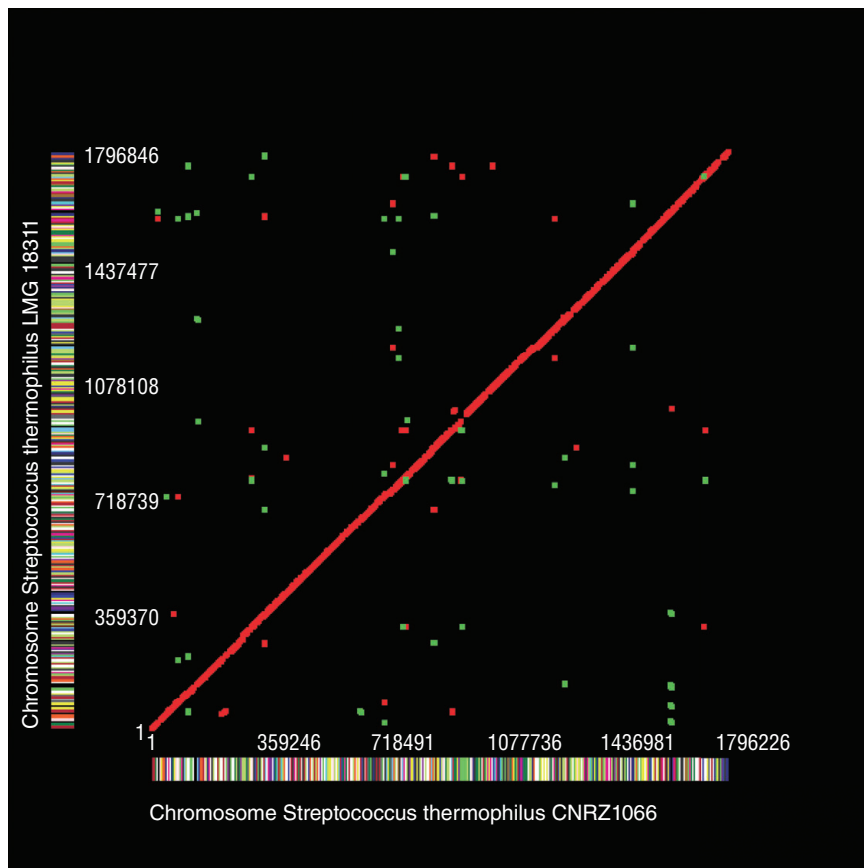


**Figure 2.4.** A. A comparison of the genomic location that contains the *pdu-cbi-cob-hem* gene cluster of *Lactobacillus reuteri* JCM 1112T (center) with the corresponding location of *Lactobacillus fermentum* IFO 3956 (upper) and *Lactobacillus plantarum* WCFS1 (lower). Genes are depicted with arrows indicating the transcription direction with the following colors: yellow, *pdu* including *gupCDE* genes; pink, *cbi* genes; orange, *cob* genes; blue, *hem* genes; red, *pocR*; green, *eut*; sky blue, transposase gene; and white, other genes. Genes conserved between the three genomes are colored gray and light blue bars indicate orthologous regions. The GC content at the third codon position of the ORFs in *Lact. reuteri* JCM 1112T is indicated under each ORF. Red lines represent the GC content at the third codon position of the ORFs in the *pdu-cbi-cob-hem* cluster (LAR\_1583-1640). The blue horizontal line indicates the average GC content (32%) at the third codon position of the remaining ORFs in the *Lact. reuteri* JCM 1112T genome excluding the *pdu-cbi-cob-hem* gene cluster. B. The *pdu-cbi-cob* gene cluster arrangement in *Lact. reuteri* JCM 1112T, *Salmonella typhimurium* LT2, *Listeria monocytogenes* EGD-e, *Yersinia enterocolitica* subsp. *enterocolitica* 8081, and *Streptococcus sanguinis* SK36 are shown using the same color coding as described in A (Morita et al. 2008). Reproduced with permission.

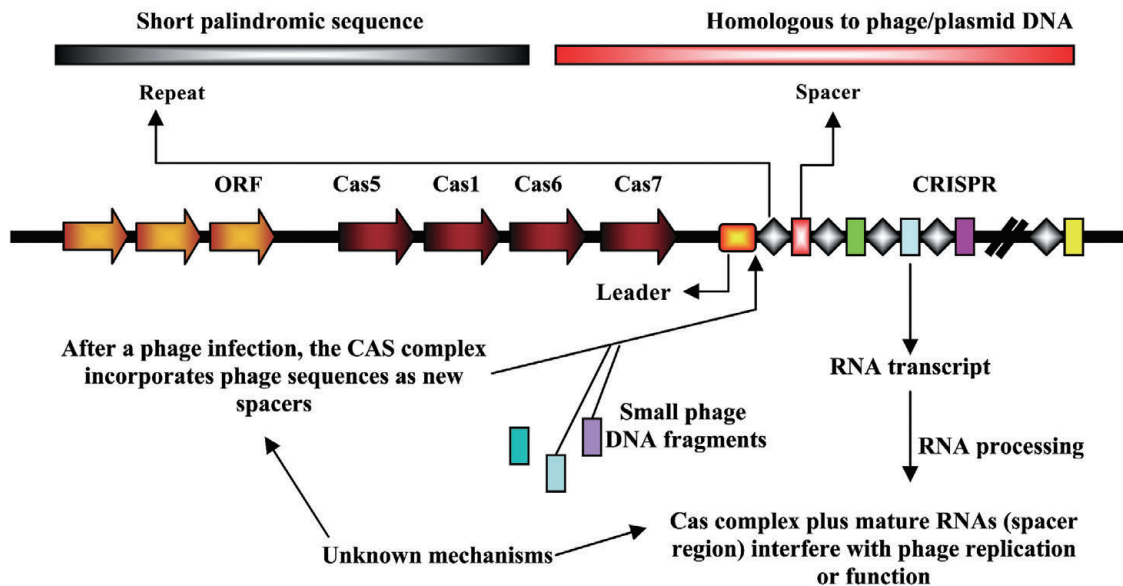




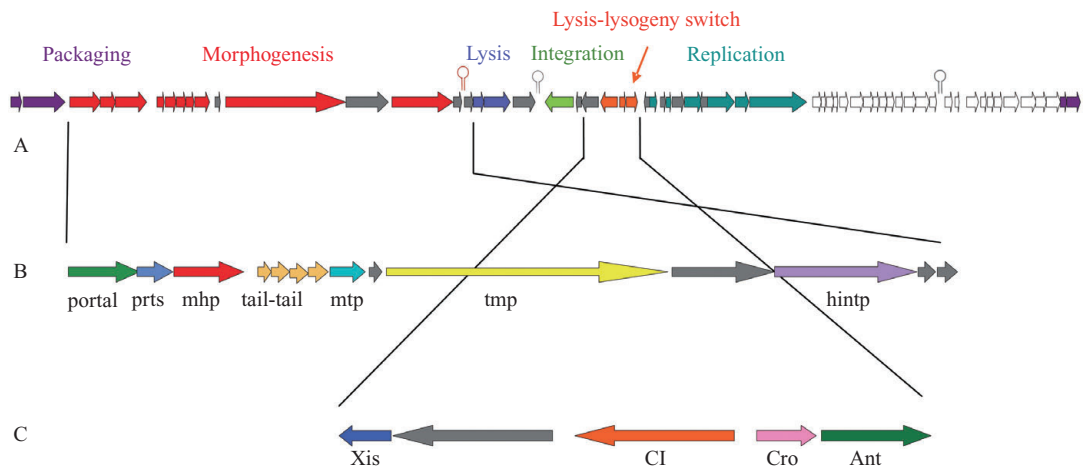
**Figure 2.5.** Organization of the genome region of selected lactic acid bacteria containing the autoinducer-2 producer gene *luxS*. The comparison was performed using the Multi-Genome Region Comparison tool in the Comprehensive Microbial Resource web site at JCVI (<http://www.jcvi.org/>). The cutoff selection for protein matches was 40% minimum percent similarity. The two chromosomal regions of *Lactobacillus delbrueckii* subsp. *bulgaricus* ATCC BAA-365 containing *luxS* are indicated.



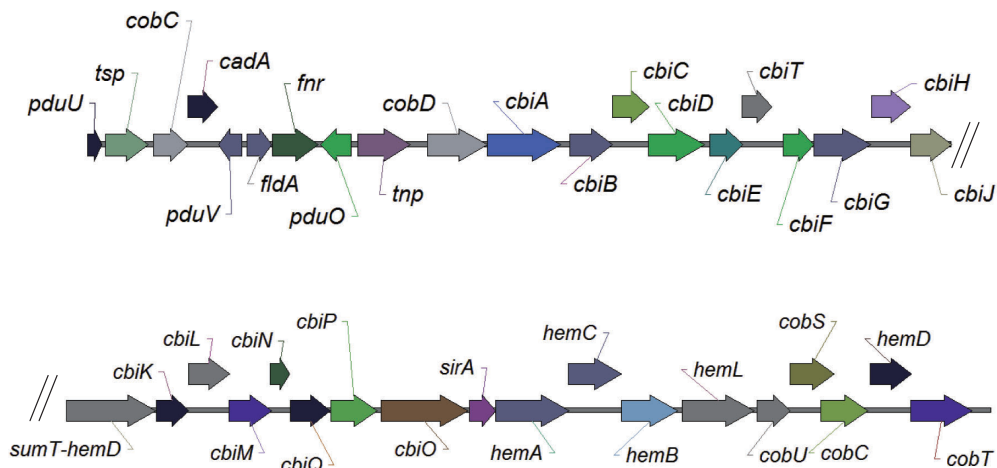
**Figure 2.6.** Whole-genome alignment of *Streptococcus thermophilus* CNRZ1066 versus *Strep. thermophilus* LMG 18311 using Maximal Unique Match (MUMmer; Kurtz et al. 2004). Minimum Match Length was set at 150. Unique regions in each chromosome are depicted as green and red dots.



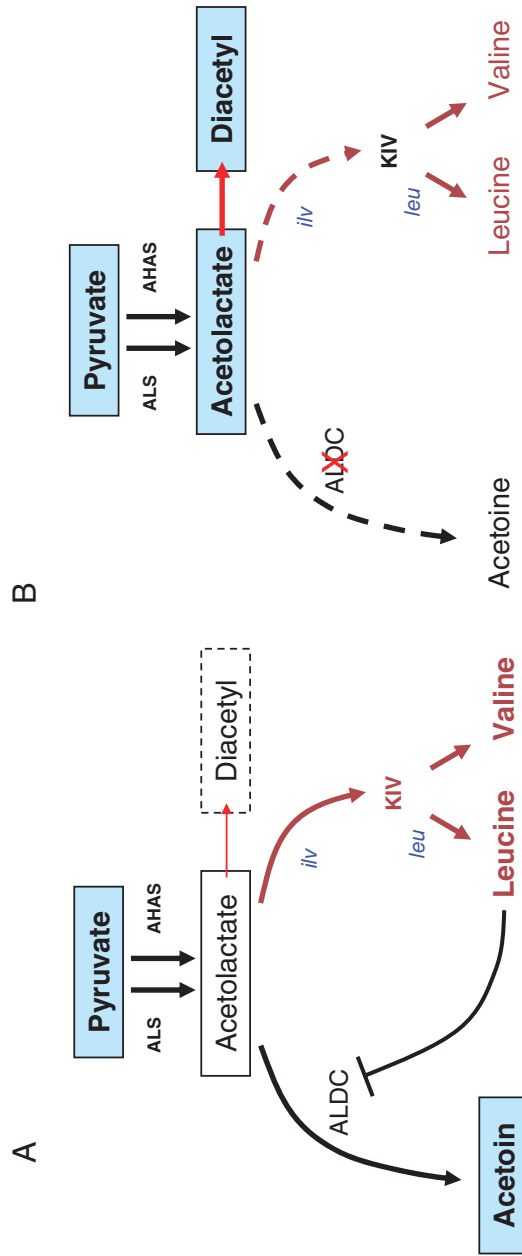
**Figure 6.1.** Scheme of a CRISPR locus.



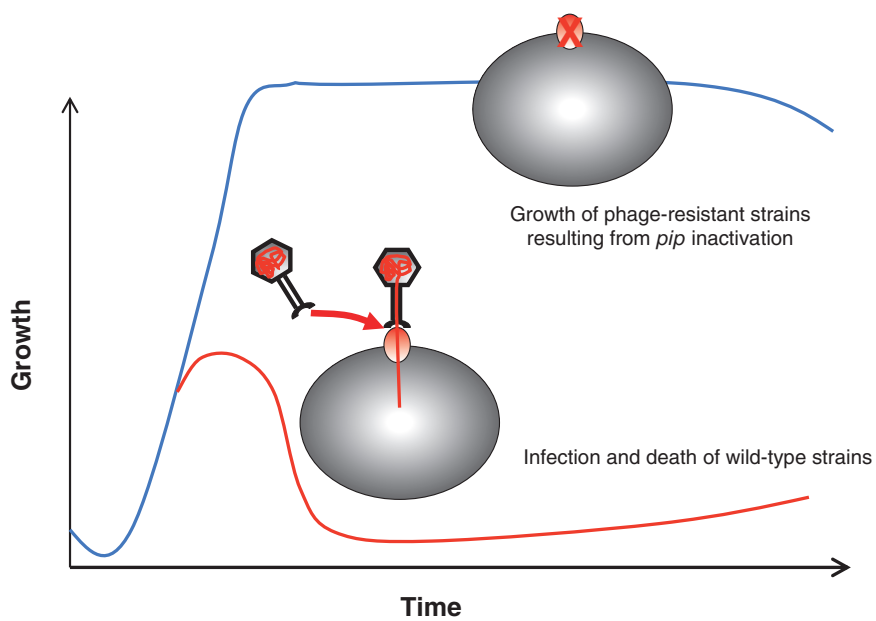
**Figure 6.3.** A. Genome sequence of phage A2. Arrows represent putatively identified genes. Modules are marked with different colors; B. morphogenesis module indicating genes and the predicted proteins; C. proteins of the lysis-lysogenic switch module: major head protein (mhp); major tail protein (mtp); protease (prts); tail measure protein (tmp); host-interaction protein (hintp); antirepressor (Ant).



**Figure 12.4** The complete vitamin B<sub>12</sub> biosynthesis gene cluster of *Lactobacillus reuteri* CRL1098.



**Figure 20.2.** Effect of *aldB* mutation in *L. lactis* on acetolactate metabolism and diacetyl production. In the wild-type strain (A) acetolactate is a precursor for acetoin and leucine and valine. The balance between the two routes is controlled by ALDC, which is allosterically triggered by leucine. In the *aldB* mutant strain, acetolactate accumulates in the medium and is available for its oxidative conversion into diacetyl.



**Figure 20.3.** Phage resistance by a single mutation the in *pip* gene. Phages are not able to attack strains carrying a mutation in the *pip* gene receptor.



## Chapter 7

# Lactic Acid Bacteria as Immunomodulators of the Gut-Associated Immune System

Carolina Maldonado Galdeano, Alejandra de Moreno de LeBlanc, Cecilia Dogi, and Gabriela Perdigón

*The safety and beneficial effects on human health of selected lactic acid bacteria (LAB) used as probiotics in many food products need to be assessed by rigorous criteria. Several studies have shown that LAB stimulate the systemic and mucosal immune systems. To establish the scientific basis for the use of these microorganisms as immune adjuvants, the elucidation of the involved mechanisms is needed. Animal model studies have demonstrated that mucosa immune activation by different LAB is mediated by the increase in the numbers of proinflammatory and regulatory cytokine producing cells in the gut, thus maintaining the intestinal homeostasis. LAB administration also showed a tendency to stimulate the Th2 response with production of systemic antibodies, bacterial viability being an important condition. Probiotic LAB can interact with epithelial cells as well as with the immune cells associated with the gut to induce immune activation; microbial cells or their cellular fragments can also be internalized by the epithelial cells. Secretory IgA, one of the principal defense antibodies at the intestinal level, is increased with the administration of probiotic bacteria. The clonal expansion of T lymphocyte population in lamina propria of the small intestine is, however, not induced by LAB. These bacteria activate mainly the innate immune response (macrophages and dendritic cells) through the increase in the expression of receptors involved in the antigen clearance and in the immune signaling as well as the reinforcement of the intestinal barrier, thus protecting the host against intestinal pathologies.*

### 7.1. Introduction

Both eukaryotic and prokaryotic cells coexist in the intestinal environment. Among the eukaryotic cells, besides the epithelial cells, the immune cells are organized forming the gut-associated lymphoid tissue (GALT). The intestinal immune cells are in contact with antigens from the external environment, the intestinal microbiota and the antigen's diet being the main antigen's stimuli. The intestinal microbiota plays an important role in the development of host's innate and adaptive immune responses and in oral tolerance mechanisms. Regulation of the composition of the gut microbiota, for example, by the use of prebiotics and probiotics, offers the possibility of influencing the development of mucosal and systemic immunity. The use of probiotics in the prevention or treatment of intestinal infections and the mechanisms by which these microorganisms affect the immune system allow the determination of the specific pathologies in which probiotic bacteria could be useful to ameliorate diseases.

### 7.2. Factors Involved in the Development of Gut Microbiota

The human intestinal microbiota includes a great number of bacteria composed by numerous genera, species, and strains. It plays an important role in human health and promotes normal gastrointestinal (GI) functions (Pickard et al. 2004). The gut microbiota exerts a barrier effect preventing the

colonization by pathogenic bacteria, and furthermore fulfills other important metabolic functions: fermentation of non-digestible fibers, source of energy as short-chain fatty acids, production of vitamin K, and stimulation of the immune system development (Guarner and Malagelada 2003). The process of colonization of the GI tract begins after birth, where the establishment of the intestinal microbiota is a complex process influenced by interactions between the host and the microorganisms, and by external and internal factors (Fanaro et al. 2003).

Digestive microbiota in newborns is developed sequentially during early life and its composition changes according to the maturation of intestinal mucosa and dietary diversification. At birth the intestine is sterile but within a few hours bacteria can be found in the faeces (Hudault 1996). The intestinal environment of neonates has a positive oxidation/reduction potential, and the GI tract is colonized first by facultative aerobes. Gradually, the oxygen is consumed by these bacteria and the intestine changes into a more reduced environment, permitting the subsequent growth of strict anaerobes (Bezirtzoglou 1997).

Recent studies demonstrated that the most important determinants of the gut microbial composition in infants depend on the delivery mode, type of infant feeding, gestational age, infant hospitalization, and antibiotic use. In this period of life, bacteria colonizing the infant gut come mainly from the mother and the environment. Vaginally born infants are colonized at first by the fecal and vaginal bacteria of the mother, whereas infants born through cesarean section are exposed initially to bacteria originating from the hospital environment and health-care workers (Bezirtzoglou 1997; Gronlund et al. 1999). Other factors that can influence the composition of the intestinal microbiota in neonates are the environment during birth, prematurity, hygienic cares, and type of infant feeding (breast or bottle feeding; Heavey and Rowland 1999). Vaginally term-born and breast-fed infants exclusively have a predominance of "beneficial" gut microbiota (highest numbers of bifidobacteria and lowest numbers of *Clostridium difficile* and *Escherichia coli*; Penders et al. 2006).

The GI tract of conventional adult mammals is constituted by a complex and dynamic community of microorganisms. The large intestine is the most intensely colonized region, with  $10^{10}$ – $10^{11}$  bacteria/g of intestinal content. The predominant species from the human colon belong to the genera *Bacteroides*, *Eubacterium*, *Bifidobacterium*, *Ruminococcus*, and *Clostridium*. Subdominant species include enterobacteria, particularly *Escherichia coli*, and streptococci (Moreau and Gaboriau-Routhiau 2000). Commensal microorganisms interact with the host without harmful effects. The intestinal immune system in healthy host allows the persistence of the normal microbiota and avoids immunological tolerance to them, maintaining the intestinal homeostasis. The presence of potentially pathogenic microorganisms in the gut is not always associated with disease. The harmful effects depend on the immune state of the host and the virulence factors of the microorganisms (Casadevall and Pirofski 2000), revealing that a complex interaction exists between microbiota and the intestinal immune system. Although the predominant human intestinal microbiota is relatively stable and unique for each individual, different environmental factors can affect the microbial composition in a transitory or permanent way (e.g., stress, diet, antibiotics treatment).

### 7.3. Influence of the Intestinal Microbiota on the GALT

The intestinal immune system is the largest and most complex part of the immune system. The main protection against potential pathogens occurs via mucosal immune system, involving mechanisms of innate immunity, as well as GALT as a secondary lymphoid organ.

GALT is poorly developed at birth; the Peyer's patches and the immune cells associated with the lamina propria in the human small intestine are present, but the cellularity is reduced. The intestinal colonization process by a complex and diverse microbiota is the most important stimulus for GALT development.

The number of IgA-secreting cells in the small intestine increases with time and with external

stimuli. Experimental model studies showed that the number of the IgA plasma cells in villous are significantly smaller in germ-free mice than in conventional mice, suggesting the important role of microbiota in the development of the intestinal immune system (Moreau et al. 1982). A similar study using gnotobiotic mice colonized with single Gram (+) or Gram (–) digestive bacteria demonstrated that different bacterial strains could affect the number of IgA plasma cells in the intestinal villous in different ways (Moreau et al. 1978).

Beneficial effects derived from the association or interaction between bacteria and the epithelial and immune cells in the intestine have been demonstrated (Isolauri et al. 2001; Kelly et al. 2005). Bifidobacteria and lactic acid bacteria (LAB) are considered the most important health-beneficial bacteria for the human host (Naidu et al. 1999). These microorganisms are commonly called “probiotics.” The concept of health-beneficial bacteria was introduced by Eli Metchnikoff (1907) to explain the link between the longevity and health of Bulgarian peasants and daily intake of fermented milk products containing non-putrefactive organisms. From these observations, Metchnikoff theorized that maintenance of a healthy gut microbiota, through daily ingestion of beneficial bacteria, was the key to a healthy long life. In fact, probiotics have been defined as “live microorganisms that when being administered in appropriate dose, they confer a benefit of health to the receiver” (FAO/WHO 2001). The health benefits induced by probiotics are related to the improvement of the normal microbiota (Rochet et al. 2008), prevention of infectious diseases (Coconnier et al. 1993, 1998; Bernet-Camard et al. 1997; Silva et al. 1999; Sheth and Garcia-Tsao 2008) and food allergies (Majamaa and Isolauri 1997; Peltto et al. 1998), reduction of serum cholesterol (Fukushima and Nakano 1996; de Roos and Katan 2000), promotion of anticarcinogenic activity (Hirayama and Rafter 1999; Reddy 1999; de Moreno de LeBlanc and Perdigon 2004; de Moreno de LeBlanc et al. 2004; Ishikawa et al. 2005), stabilization of the gut mucosal barrier (Salminen et al. 1996), increase in immune adjuvant properties (Schiffrin et al. 1995; Fukushima et al. 1998; Kato

et al. 1999; Yasui et al. 1999; Gill et al. 2000; Isolauri et al. 2001; de Moreno de LeBlanc et al. 2005a; Di Giacinto et al. 2005), alleviation of intestinal bowel disease symptoms (Schultz and Sartor 2000; Herias et al. 2005), and improvement of lactose digestion (de Vrese et al. 2001; Kopp-Hoolihan 2001).

The immunological properties of probiotic bacteria have been extensively studied; certain LAB such as *Lactobacillus casei*, *Lactobacillus rhamnosus*, and *Lactobacillus plantarum* enhance both systemic and mucosal immunity (Perdigon et al. 1999, 2001). Furthermore, *Lactobacillus acidophilus* strain La1 (able to adhere to enterocytes) and *Bifidobacterium bifidum* strain Bb12, which shows high fecal colonization, stimulate the phagocytic cells more efficiently than other bacteria (Schiffrin et al. 1997). Studies on the intestinal mucosal immune system showed that not all LAB could be used as oral adjuvants and that their beneficial effects could not be related to bacterial genera or species, because strain variations may occur (Vintiñi et al. 2000).

#### 7.4. Probiotic Interaction with Epithelial and Immune Cells in the Small Intestine

The correct use of probiotics requires understanding of the mechanism by which these microorganisms interact with the intestinal immune cells. Criteria for probiotic selection include their resistance to the enzymes in the oral cavity (amylases and lysozyme), to the low pH in the stomach, and to the concentration of bile and pancreatic juices of the small intestine. Studies conducted with viable and non-viable probiotic bacteria labeled with fluorescein isothiocyanate, administered to mice by intragastric intubation, showed that bacterial viability is an important condition for bacterial adhesion to intestinal cells, this first contact between probiotic bacteria and epithelial cells being crucial in following events (Galdeano and Perdigon 2004). Viable bacteria were observed up to 72 h while dead bacteria were rapidly depurated by the immune cells.

As any other particulate antigen, probiotic microorganisms must also be able to persist in the GI tract and to interact with the gut epithelial tissue. The probiotic bacterium *Lact. casei* CRL431, for example, adhered to the gut epithelial cells of BALB/c mice after oral administration (Galdeano and Perdigon 2004). Immunolabeling assays by electron microscopy using colloidal gold particles showed that the interaction of this probiotic bacterium with intestinal epithelial cells occurs after 5 min of oral administration. The entire bacteria were present in the lumen of the intestine or adhered to the apical surface of the epithelial cells, while labeled cell wall antigen particles were only detected inside the intestine cells. The colloidal gold antigen particles were found both in the cytoplasm and in the intercellular spaces of the epithelial cells of the small intestine. The uptake of particulate antigens, macromolecules, and microorganisms can occur by active transepithelial vesicular transport, with the enterocytes being able to endocytose small amounts of intact proteins and peptides across the epithelium (Neutra and Kraehenbuhl 1993). In accordance with this, the colloidal gold particles observed in the intercellular spaces may be internalized by this process.

Other potential cells involved in the internalization process are M and dendritic cells. M cells are distinctive epithelial cells found in the follicle-associated epithelium of the Peyer's patches that play a key role because of their capacity to transport macromolecules, microorganisms, and inert particles from the lumen into the lymphoid tissue by adsorptive endocytosis. M cells are not the only type of cells capable of transporting antigens across the epithelial barrier. Dendritic cells (DC) might extend their dendritic-like projections through epithelial tight junctions and sample luminal antigens directly from the small intestine as reported by Rescigno et al. (2001). During this process the integrity of the epithelial barrier would be maintained due to tight junctions constituted by proteins expressed on both enterocytes and DC.

The epithelial cells are in contact with the antigens present in the lumen and they respond to environmental signals by releasing chemokines and

cytokines by the immune cells from both innate and adaptive immune responses. The interaction of the probiotic bacterium *Lact. casei* CRL431 with isolated intestinal epithelial cells was reported to be mediated by Toll-like receptor-2 (TLR-2), producing an increase in IL-6 in the culture supernatant (Vinderola et al. 2005).

## 7.5. Probiotics: Activation of the Intestinal Immune System and the Innate Immune Response in the Gut

In recent years the consumption of dietary supplements containing probiotic bacteria has increased notably based on the idea that probiotics help to maintain a beneficial balance of the intestinal microbiota. The main probiotic vehicles are fermented products, with bifidobacteria and lactobacilli being the principal species involved. Probiotics have been reported to stimulate both innate and adaptive immune response by increasing macrophages (MC) activation and antibody production (Sato et al. 1988; Haller et al. 2000; Kato 2000). Children and elderly populations have often been the subject of studies to determine the clinical effects of probiotics, because these groups are more susceptible to infection compared with young adults. It was reported that children attending a day-care center who were supplemented with *Lact. rhamnosus* GG showed a significantly reduced incidence of respiratory infections (Hatakka et al. 2001). Similar beneficial effects in the control or prevention of diarrhea with other probiotic strains have been demonstrated (Szymanski et al. 2006; Binns et al. 2007), which suggest that the enteropathogenic infection in children may be ameliorated by continuous supplementation of probiotics to the diet.

On the other hand, Turchet et al. (2003) showed that the duration of winter infections in the elderly population was shorter in subjects administered with fermented milk containing *Lact. casei* DN-114001 than in controls without fermented milk supplementation. Another study demonstrated that dietary supplementation with *Lactobacillus johnsonii* La1 reduced the duration of respiratory infection

(Fukushima et al. 2007). However, the mechanisms of how supplementation with probiotics can reduce the incidence of respiratory tract infection remain to be clarified. It has been suggested that the increase in the number and functionality of natural killer (NK) cells could explain those effects, probably by increasing the antibody levels as was described after influenza vaccination (de Vrese et al. 2006; Olivares et al. 2007). When the adjuvant capacity of different probiotics was studied in animal models, *Lact. casei* CRL431, in particular, was able to protect against *Salmonella enteritidis* serovar Typhimurium and *Escherichia coli* infections in mice, through an increase in the specific S-IgA into the intestinal lumen (Perdigon et al. 1991; Gobbato et al. 2008).

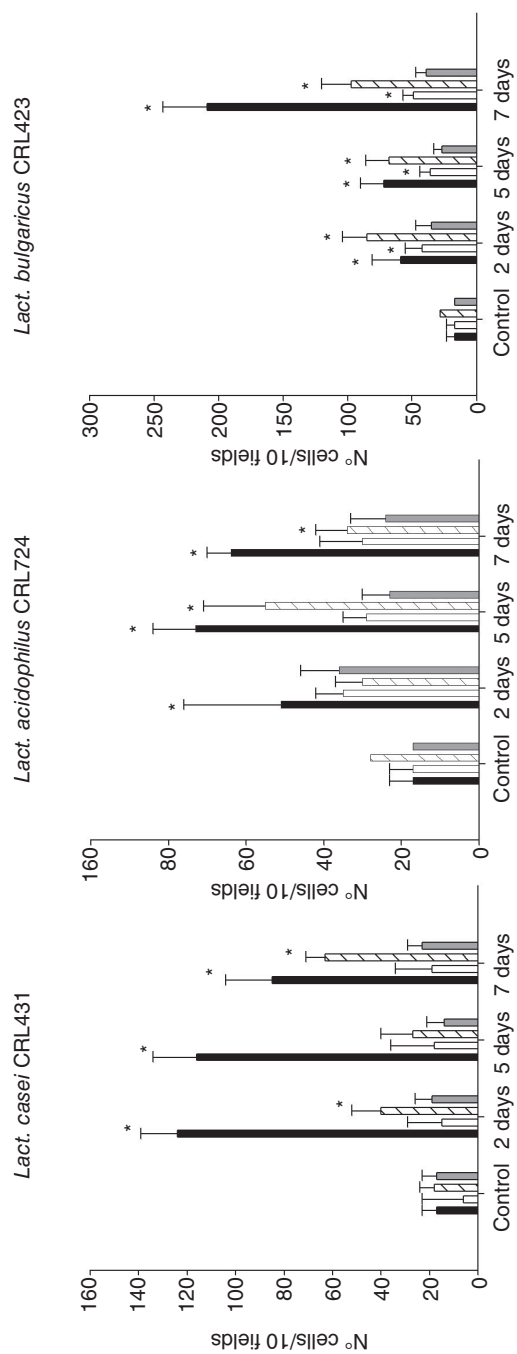
Cytokine production is a consequence of the immune cells activation; the profile obtained allows predicting the type of response (regulatory or inflammatory). In fact, cytokines are considered biological messengers that are able to balance the immune response. Oral administration of mice with different LAB stimulated the number of cytokine-positive cells in lamina propria and in Peyer's patches of the small intestine, the effect being dose-dependent (Perdigon et al. 2002b). LAB were able to induce the production of inflammatory cytokines such as TNF $\alpha$  and IFN $\gamma$ , as well as regulatory cytokines such as IL-4 and IL-10. The observed cytokine profile varied according to the LAB administrated (Maassen et al. 2000; Perdigon et al. 2002b) and was dependent on the viability of the LAB strains used; viable cells elicited a better immune stimulation. The effect of bacterial viability was more remarkable with *Lact. casei* and *Lact. acidophilus* strains than with *Lact. delbrueckii* subsp. *bulgaricus* and particularly for the cytokines IFN $\gamma$  and IL-10 (Fig. 7.1).

Oral administration of probiotics may also stimulate mucosal sites distant to the intestine. Thus, the oral administration of the probiotic strain *Lact. casei* CRL431 to mice produced an important increase in the number of IgA+ cells in bronchus and mammary glands (de Moreno de LeBlanc et al. 2005a). *Lact. casei* CRL431 administration was not able to induce T cell proliferation in the lamina propria of the small

intestine in either CD4 or CD8 T cells. These results led to the inference that this LAB strain could be used as an oral adjuvant mucosal surface; it is also useful against mammary gland and respiratory pathologies (Fig. 7.2).

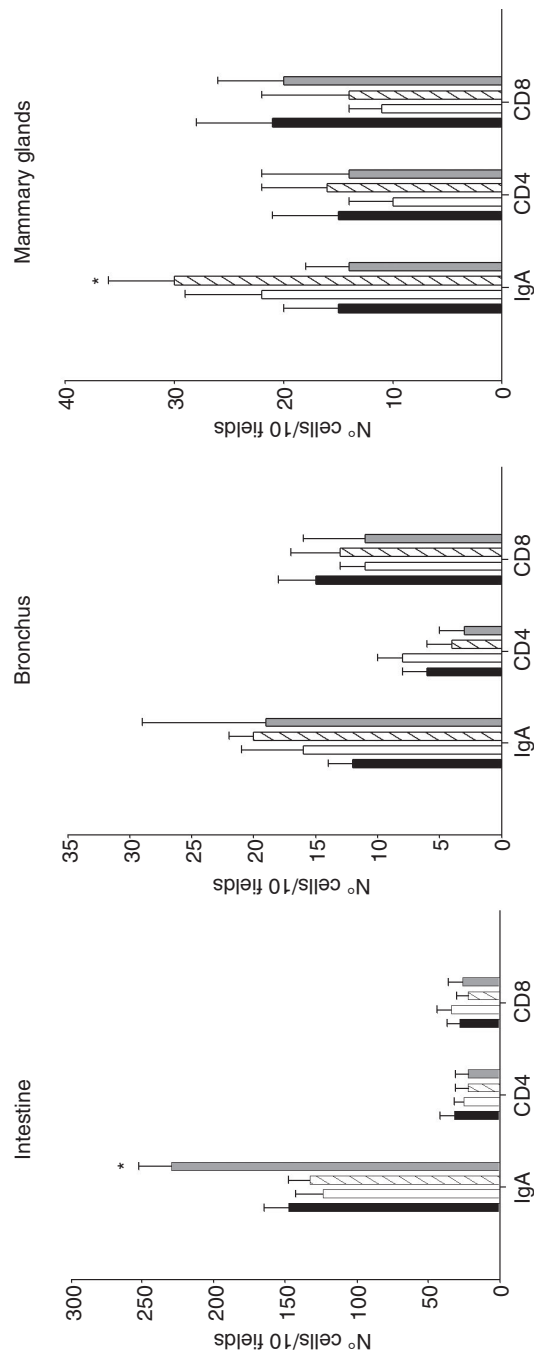
*Ex vivo* studies demonstrated that the main cells involved in cytokine production such as TNF $\alpha$  were the phagocytic cells: MC and DC (Perdigon et al. 2002b). Proliferation of T cells after the probiotic administration was not observed, suggesting that the main cells involved in the immune stimulation by probiotic bacteria were those involved in the innate immune response. Phagocytic cells express pattern-recognition receptors such as TLRs, C-type lectin receptors and Nod-like receptors that are specific for various microbial components. These patterns of recognition receptors expressed by MC and DC of the GALT can recognize the probiotic bacteria components. One of the most intensively studied families of pattern recognition receptors is the TLR family. TLRs play a central role in alerting antigen-presenting cells to the presence of pathogenic bacteria (Dunzendorfer et al. 2004). TLRs can activate the innate immune response, mainly inflammatory, before the adaptive immune response (Ahmad-Nejad et al. 2002; Heil et al. 2003; Matsumoto et al. 2003). Among this family of TLRs, TLR-2 recognizes a variety of microbial components such as lipoproteins/lipopeptides from various pathogens, peptidoglycans, and lipoteichoic acid from Gram (+) bacteria. It has been reported that TLR-2 is also able to recognize lipopolysaccharide preparations from enterobacteria such as *Leptospira interrogans*, *Porphyromonas gingivalis*, and *Helicobacter pylori* (Kalliomaki et al. 2001; Turchet et al. 2003).

*In vitro* studies demonstrated that *B. bifidum* W32 and *Bifidobacterium infantis* W52 stimulated TLR-2 in ovary cells and activated the transcriptional factor NF-kB (Niers et al. 2007). Another study performed with lipoteichoic acid derived from *Lact. casei* Shirota showed the stimulation of MC to secrete TNF $\alpha$  via TLR-2 signaling (Matsuguchi et al. 2003). Orally ingested *Lact. rhamnosus* Lr23 could promote the development of regulatory DCs in a TLR-2-dependent manner (Foligne et al. 2007).



**Figure 7.1.** Effect of viable and nonviable LAB on the number of IFN $\gamma$  and IL-10 positive cells in lamina propria of the small intestine. Cytokine positive cells were determined in mice receiving the viable (black and diagonal line bars for IFN $\gamma$  and IL-10, respectively) and nonviable (white and gray bars for IFN $\gamma$  and IL-10, respectively) LAB ( $2 \times 10^9$  UFC ml $^{-1}$ ) in the drinking water during 2, 5, or 7 days. Cells were analyzed by indirect immunofluorescence on small intestine. Values are expressed as mean  $\pm$  S.D. of number of positive cells (fluorescent cells) counted in 10 fields of vision at 1000x magnification (cells/10 fields) using a fluorescence light microscope. \*Significant differences between groups and control ( $P < 0.05$ ).





**Figure 7.2.** Determination of the number of IgA+, CD4+ and CD8+ cells in the intestine, bronchus, and mammary glands after *Lact. casei* CRL431 administration. Cells were determined on histological sections of control group (black bars) and mice fed with *Lact. casei* CRL431 during 2 (white bars), 5 (diagonal line bars), and 7 (gray bars) days by direct immunofluorescence assays. The values are expressed as mean  $\pm$  S.D. of number of positive cells (fluorescent cells) counted in 10 fields of vision at 1000x magnification (cells/10 fields) \*Significant differences between groups and control ( $P < 0.05$ ).

Also, the number of TLR-2 as well as CD-206 positive cells (see below), in both lamina propria and Peyer's patches of mice, significantly increased after oral administration of *Lact. casei* CRL431 (Galdeano and Perdigon 2006). Moreover, *in vitro* assays showed that *Lact. casei* CRL431 stimulates IL-6 release by intestinal epithelial cells through TLR-2 (Vinderola et al. 2005). These results indicate the central role of TLR-2 in the interaction between probiotics and immune cells. The fact that antigen particles from probiotic bacteria can interact with the intestinal immune cells suggests that TLR-9 could also be involved in the signal process to activate the immune cells of the gut (Dogi et al. 2009).

Another family of receptors involved in pattern recognition is the mannose receptor family. The mannose receptor CD-206 binds carbohydrate groups containing mannosyl/fucosyl residues and a terminal lectin domain that binds sulfated carbohydrate groups and is able to recognize a number of microbial proteoglycans. This receptor is involved in the clearance of self-antigens such as endogenous proteins, including myeloperoxidase, lysosomal hydrolases, and some hormones that contain sulfated carbohydrate groups (Lee et al. 2002). As stated above, the number of positive cells for this receptor in mice was significantly increased after *Lact. casei* CRL431 administration. The increase in CD-206 and TLR-2 could be due to upregulation of these markers, in agreement with previous studies (Galdeano and Perdigon 2004) where probiotic bacteria or their antigenic particles internalized by DC or MC induced the increase of these two receptors.

## 7.6. Mechanisms of Immunomodulation by Probiotics in the Gut

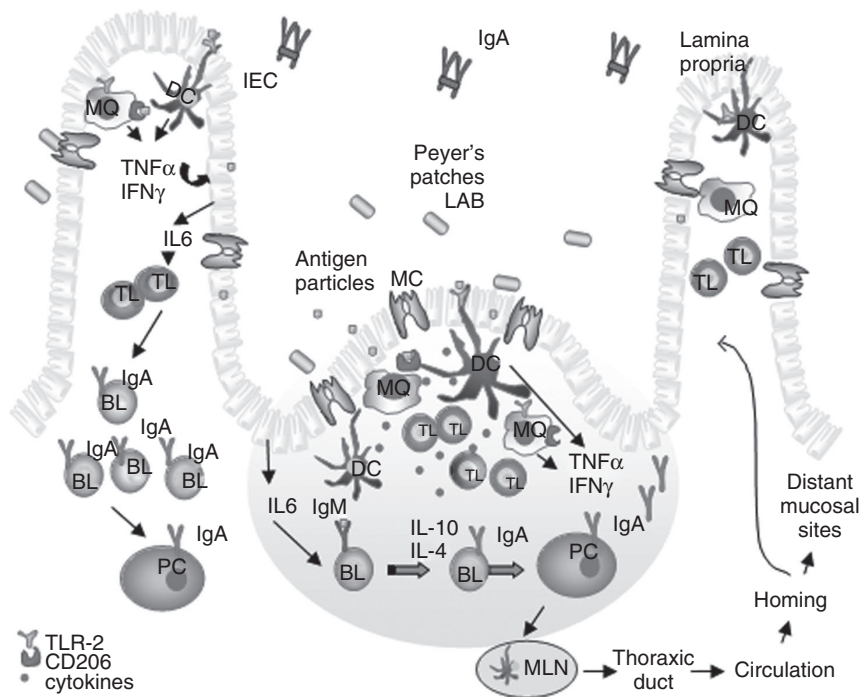
Based on the results mentioned above, a model for the interaction between the probiotic strain *Lact. casei* CRL431 with the intestinal epithelial and immune cells that have in consideration the effects of these interactions on the gut mucosal immune system was proposed (Maldonado Galdeano et al. 2007; Fig. 7.3).

It is proposed that probiotic bacteria in the gut lumen are recognized and processed by the immune

system through several routes. The adherence of these microorganisms to the epithelial cells, through TLR-2 receptors, induces the release of IL-6 by intestinal epithelial cells. Probiotics may also be internalized by the gut immune cells to trigger and modulate the immune function in the intestine as well as by DC and M cells. DC might directly sample and process probiotics in the gut lumen through interdigitant projections, while M cells located in the Peyer's patches or in the villous may take probiotics up directly by transcytosis (Neutra and Kraehenbuhl 1993).

MC and DC are the first immune cells that can interact with the probiotic or their cellular fragments. These cells can recognize the antigens through the receptors TLR-2 and CD-206 with the consequent production of cytokines such as TNF $\alpha$ . This is the main cytokine participating in the cross talk between the intestinal and immune cells. MC and DC exposed to probiotics can secrete a variety of cytokines, such as IL-10 and IL-6, enhancing the network of cytokine signals. Other cytokines produced are IL-12 and IL-10, which control the balance of the immune response; IL-12 increases cellular immunity whereas IL-10 induces down immune regulation responses. IL-6 together with IL-4 and Transforming Growth Factor- $\beta$  (TGF- $\beta$ ) may induce the T-independent switch from IgM to IgA on the surface of B lymphocytes, promoting an increase in the number of IgA+ B lymphocytes in the lamina propria of the gut. It was reported that the number of IgA+ cells in the lamina propria of the small intestine increased after administration of different LAB, such as *Lact. acidophilus*, *Lact. bulgaricus*, and *Streptococcus thermophilus*, probably by IL-6 and IL-5 produced by immune cells (Galdeano and Perdigon 2006).

Probiotic stimulation can also induce the IgA cycle by increasing the number of IgA+ cells in mucosal sites distant of the intestine. The IgA+ cells migrate from Peyer's patches to the mesenteric lymphoid node and then via the thoracic duct to the circulation, arriving to the bronchus and mammary glands. The cytokines released by probiotic stimulation in Peyer's patches are the biological messengers of the complex network of signals to induce cell



**Figure 7.3.** *Lact. casei* CRL431 and epithelial and immune cells interaction model in the small intestine. Three hypothetical pathways by which probiotics interact with the immune cells and modulate immune function in the intestine are proposed. (1) Probiotics contact directly with the intestinal epithelial cells (IEC), which secrete cytokines such as IL-6 and initiate the dialog with adjacent immune cells; (2) specialized epithelial cells called M cells (MC) present in the Peyer's patches or in the villi. Macrophages (MQ) or dendritic cells (DC) are the first cells immediately below M cells taking contact with probiotic bacterium or their antigen fragments and producing cytokines; and (3) DC in the intestinal lamina propria has been found to extend their dendrites between IEC and might directly sample and process probiotics in the gut lumen.

migration and to activate the systemic immune response (Lamm 1976). Specific IgA against the probiotic bacteria epitopes and modifications in the number of CD4+ or CD8+ populations were not found when probiotic bacteria were administered by oral route (Galdeano and Perdigon 2006). These findings suggest that antigen presentation with production of specific antibodies anti-probiotic bacteria would not be induced.

In conclusion, the activation of the gut immune system induced by the probiotic bacterium *Lact. casei* CRL431 denotes that the main immune cells implicated are those involved in the innate immune response (MC and DC) while the T cell population is less involved in the observed immune modulation (Galdeano and Perdigon 2006).

## 7.7. Consumption of Probiotic Bacteria Suspensions or Fermented Milks: Effects on the Intestinal Immune System

### 7.7.1. Administration of Fermented Milks

The intake of fermented products and their relationship with host health was first reported a century ago. Many researchers have studied the beneficial effects of yogurt and LAB commonly used in yogurt production against infections, GI disorders, and cancer (Adolfsson et al. 2004; Parvez et al. 2006). In addition to LAB, fermented milks possess other non-bacterial components produced during the fermentation that can contribute to immunogenicity and other nutritional properties. Peptides and free

fatty acids released during milk fermentation were shown to increase the immune response (Meydani and Ha 2000). Fractions separated by dialysis from yogurt showed tumor inhibition in *in vivo* murine assays (Ayebo et al. 1982). Certain soluble components produced by LAB during milk fermentation could be used to prevent some malignant pathology (Biffi et al. 1997). Also, it was reported that the filtrate of yogurt increased IFN $\gamma$  production by NK cells (De Simone et al. 1986).

Anti-mutagenic and immunomodulator properties were described for a milk fermented with *Lactobacillus helveticus* R389 (Matar et al. 1997, 2001). Both beneficial effects were related to the high proteinase and peptidase enzyme activities of the wild-type strain R389, since they were not detected in milk fermented with the proteolytic deficient mutant. The number of IgA+ cells was increased both in the small intestine and in the bronchus of mice fed with the milk fermented by strain R389 (Matar et al. 1997, 2001). Peptidic fractions liberated during milk fermentation with *Lact. helveticus* R389 stimulated the immune system and inhibited the growth of an immune-dependent fibrosarcoma in an experimental mice model (LeBlanc et al. 2002). The consumption of this fermented milk also decreased the growth of a breast tumor in a murine model (de Moreno de LeBlanc et al. 2005b, 2005c).

### 7.7.2. Administration of Conventional Yogurt

Conventional yogurt is one of the most consumed fermented products in Western populations. The immunomodulatory capacity of yogurt and other fermented milks is one of the best documented effects attributed to these products. Administration of commercial-type yogurt to BALB/c mice induced a marked immune cell infiltration with plasma cells and lymphocyte prevalence (Perdigon et al. 1994) when it was given during short periods of time. Yogurt feeding for 7 days increased the number of IgA+ cells in the small and large intestine of mice but IgM+ and IgG+ cells were not increased (Perdigon et al. 1998). Macrophage numbers were also increased compared with the non-treated group,

but they did not show an increased activity. The increase of IgA but not IgG could explain why yogurt does not induce the undesirable inflammatory response (Valdez et al. 1997).

It was also reported that cyclic yogurt feeding inhibited the development of an experimental colon cancer induced by dimethylhydrazine in BALB/c mice. Before tumor development, the animals exhibited a significant inflammatory response with mononuclear cells infiltration in the large intestine. When these mice were cyclically fed with yogurt, both the tumor growth and the inflammatory response were inhibited (de Moreno de LeBlanc and Perdigon 2004, 2005). These results were obtained in an experimental model where mice received yogurt during a prolonged (6 months) period of time. This long-term feeding of yogurt increased the number of IgA+ cells in the large intestine, values that were maintained in high number during yogurt administration. An increase in the CD4+ T lymphocyte population was only observed in mice that received yogurt in the presence of the carcinogen; in contrast, higher numbers of CD8+ and IgG+ were detected in the tumor control group (Perdigon et al. 2002a). The analyses of different immune cell populations suggested the possible anti-inflammatory role of yogurt (also related to the anticarcinogenic effect of this product).

Cytokines are important biological messengers in the stimulation, regulation, and modulation of the immune response. The long-term cyclic yogurt feeding significantly increased, compared with the non-treated group, the levels of cytokine TNF $\alpha$  an apoptosis mediator produced by monocyte-macrophages infiltrated in the tumor, and IFN $\gamma$  a cytokine produced by CD4+ cells (de Moreno de LeBlanc and Perdigon 2005). However, since the inducible oxide nitric synthase enzyme (iNOS) was not induced in the cells of the large intestine of yogurt-fed mice and no inflammation was observed, the increase of these cytokines was not related to inflammation (Table 7.1). As expected, the levels of iNOS, an enzyme induced by microbial products and cytokines during the immune response displaying an important role in the antimicrobial mechanisms exerted by M cells (de Moreno de LeBlanc et al.

**Table 7.1.** Comparative study of the cytokine positive cells and iNOS enzyme in the intestine of mice fed with fermented milks or probiotic bacterium.

Experimental group	Intestine section	Feeding period	TNF $\alpha$	IFN $\gamma$	iNOS	IL-10	IL-4
Yogurt	LI	10 days	29 $\pm$ 3	25 $\pm$ 3	8 $\pm$ 2	23 $\pm$ 2	14 $\pm$ 2
		3 months	50* $\pm$ 7	100* $\pm$ 10	19* $\pm$ 5	62* $\pm$ 9	26 $\pm$ 4
		4 months	57* $\pm$ 8	112* $\pm$ 9	11* $\pm$ 2	57* $\pm$ 12	33 $\pm$ 7
		5 months	81* $\pm$ 9	129* $\pm$ 14	14* $\pm$ 3	55* $\pm$ 6	17 $\pm$ 2
		6 months	101* $\pm$ 14	86* $\pm$ 17	12* $\pm$ 2	62* $\pm$ 12	25 $\pm$ 3
CRL431	SI	2 days	50* $\pm$ 6	99* $\pm$ 11	ND	41* $\pm$ 2	90* $\pm$ 5
		14 days	58* $\pm$ 15	90* $\pm$ 3	ND	79* $\pm$ 4	186* $\pm$ 9
		28 days	75* $\pm$ 23	124* $\pm$ 13	ND	76* $\pm$ 4	222* $\pm$ 19
		56 days	55* $\pm$ 18	116* $\pm$ 7	ND	33 $\pm$ 3	97* $\pm$ 7
		84 days	44* $\pm$ 4	116* $\pm$ 18	ND	32 $\pm$ 3	132* $\pm$ 10
		98 days	45* $\pm$ 2	96* $\pm$ 1	ND	32 $\pm$ 2	128* $\pm$ 4
Probiotic fermented milk	SI	2 days	96* $\pm$ 13	47* $\pm$ 2	ND	98* $\pm$ 8	ND
		14 days	75* $\pm$ 15	38* $\pm$ 4	ND	64* $\pm$ 17	ND
		28 days	91* $\pm$ 8	51* $\pm$ 6	ND	68* $\pm$ 6	ND
		56 days	73* $\pm$ 11	38* $\pm$ 3	ND	91* $\pm$ 7	ND
		84 days	35 $\pm$ 4	42* $\pm$ 8	ND	62* $\pm$ 6	ND
		98 days	42 $\pm$ 5	47* $\pm$ 8	ND	61* $\pm$ 5	ND
	LI	2 days	35* $\pm$ 10	31* $\pm$ 8	ND	50* $\pm$ 7	ND
		14 days	67* $\pm$ 8	28* $\pm$ 4	ND	48* $\pm$ 4	ND
		28 days	70* $\pm$ 7	30* $\pm$ 4	ND	40* $\pm$ 10	ND
		56 days	23 $\pm$ 3	28* $\pm$ 2	ND	52* $\pm$ 6	ND
		84 days	22 $\pm$ 3	35* $\pm$ 12	ND	39* $\pm$ 5	ND
		98 days	23 $\pm$ 2	22 $\pm$ 4	ND	25 $\pm$ 3	ND
	SI		30 $\pm$ 7	25 $\pm$ 4	ND	31 $\pm$ 4	34 $\pm$ 2
		LI	23 $\pm$ 3	21 $\pm$ 2	10 $\pm$ 2	22 $\pm$ 3	20 $\pm$ 3

Mice received yogurt or the fermented milk containing the probiotic *Lact. casei* DN 114001 (PFM) *ad libitum* during 6 months or 98 days, respectively. *Lact. casei* CRL431 was administered in the drinking water (doses,  $2 \times 10^9$  UFC ml $^{-1}$ ) during 98 days. Cells were analyzed by indirect immunofluorescence on small intestine (SI) or large intestine (LI) tissues. Results are expressed as number of positive cells for the corresponding cytokine or enzyme in 10 fields of vision as seen at 1000 $\times$  magnification using a fluorescence light microscope.

For the control of both parts of the intestine only one datum is expressed because no significant differences were observed for this group of mice in the different time point assayed.

\*Represent a significant difference ( $P < 0.05$ ), compared with the respective control of SI or LI. ND = Not determined.

2004), were increased in mice bearing intestinal tumor. Moreover, long-term yogurt feeding increased the number of positive cells for the regulatory cytokines IL-10 and IL-4 (Table 7.1); these two cytokines inhibited IFN $\gamma$  production by activated T cells. Also, IL-10-deficient mice showed high incidence of colorectal carcinomas as reported by Berg et al. (1996).

The increase of the cytokines IFN $\gamma$ , TNF $\alpha$ , IL-10 and IL-4 and the lack of iNOS enzyme induction in mice fed with yogurt suggest that yogurt may regulate the immune system by modulating the inflammatory response. Thus, long-term yogurt feeding

stimulated and increased the number of the immune cells associated with the intestine. No inflammatory response was observed in mice receiving yogurt showing a modulated response with increase in the number of regulatory cytokines that could be related with the anti-inflammatory effects attributed to the yogurt consumption.

### 7.7.3. Administration of *Lact. casei* CRL431

The probiotic bacterium *Lact. casei* CRL431 is being used in fermented products with proven health

benefit properties on the host. The immunomodulatory capacity of this strain was previously described in this chapter using a mouse model fed with *Lact. casei* CRL431 for short periods of time. The cyclical administration of this strain to mice during 98 days showed the stimulation of the mucosal immunity with homeostasis maintenance in the gut (Bibas Bonet et al. 2006); no side effects such as inflammatory immune response even under constant antigenic stimulation were observed. Only a slight increase in the cellularity in the villous and lamina propria of the small intestine was detected.

*Lact. casei* CRL431 increased the number of IgA+ cells in the small intestine (Table 7.1). The administration of this probiotic bacterium also increased the TNF $\alpha$ - and IFN $\gamma$  producing cells up to 28 days; a significant increase in the regulatory cytokines such as IL-10 was also observed. After this period, the number of positive cells for this cytokine remained similar to those of the control. In contrast, a constant increase of IL-4 in the immune cells of the small intestine was observed during the complete experimental period.

#### 7.7.4. Administration of Fermented Milk Containing *Lact. casei* DN-114001

The beneficial effects of the consumption of fermented milk containing the probiotic strain *Lact. casei* DN-114001 were reported (Turchet et al. 2003; Medici et al. 2005). The modulator effect of the long-term administration of this fermented milk on the intestinal immune system as well as the protection of the distant mucosal tissues such as bronchus and mammary glands were ascribed to the increase in the IgA cycle (de Moreno de LeBlanc et al. 2008). At the intestinal level, the continuous consumption of a milk fermented by this probiotic strain increased IL-2 positive cells as well as the number of TNF $\alpha$  and IFN $\gamma$  positive cells in the large and small intestine; however, a decrease in the number of TNF $\alpha$  positive cells was observed at the end of the experiment (de Moreno de LeBlanc et al. 2008). The IL-2 cytokine is involved in the progression of T lymphocytes as a growth factor (Feghali and Wright 1997) and is produced by T lymphocytes and

DC (Rizza et al. 2002). The increase in IL-2-producing cells is correlated with the increased number of T cells found in both intestines (de Moreno de LeBlanc et al. 2008) as well as with the number of other cytokine positive cells (Table 7.1). No inflammation was observed in mice given the fermented milk, suggesting that TNF $\alpha$  and IFN $\gamma$  may produce other effects in the intestine (such as apoptosis mediated by TNF $\alpha$ ) or that the proinflammatory effect of these cytokines could have been modulated. This latter effect could be attributed to the regulatory IL-10, which was significantly increased with respect to the control values throughout the experiment (de Moreno de LeBlanc et al. 2008).

## 7.8. Conclusions

The effects of probiotic LAB, delivered as suspensions or fermented milks, on the gut mucosal immune system have been described. Selected probiotic LAB strains stimulated the mucosal immunity, particularly at the intestinal level without modification of the intestinal homeostasis. This stimulation may be produced by the antigen stimuli from the microorganisms and/or from other non-bacterial component present in the fermented products. Also, the stimulation of some beneficial intestinal microbiota could affect the immune cells associated with the intestine. It is well known that the beneficial effects of probiotics are strain-dependent and not specific for any particular genera or species, and that several beneficial effects are not specific for a single bacterium. The probiotic bacteria and/or selected fermented products could be useful as adjuvant of the mucosal immune system, promoting an increase in the number of IgA+ cells. These cells are the first line of defense against infections; the S-IgA antibodies are the major effector molecules in the mucosal system.

The long-term consumption of probiotic fermented milks may contribute to the maintenance of the surveillance mechanism against harmful stimuli that enter the intestine without affecting the homeostasis of the gut ecosystem. The induction of regulatory cytokines such as IL-10 and IL-4 and the



absence of inflammatory reaction are also desirable. Human trials are necessary to validate the mucosal adjuvant effect observed in animal experimental models and to demonstrate the hypothesis that continuous consumption of probiotic fermented milks can favor gut surveillance mechanisms against intestinal pathologies or infections.

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## Chapter 8

# Lactic Acid Bacteria in the Prevention of Urogenital and Respiratory Infections

María E. Fátima Nader-Macías, Gladis Susana Alvarez, Clara Silva de Ruiz, Marcela Medina, and María Silvina Juárez Tomás

*During the last decades there has been an explosion in the number of publications related to effects of probiotic microorganisms in the gastrointestinal tract, both of humans and animals. More recently, and stimulated by the need for new strategies to increase or maintain the “high quality of life” parameters, the application of probiotics is spreading to diverse areas such as the urogenital and respiratory tracts. The use of probiotics in these tracts is being studied to prevent infections and to improve the health status of the host. Urogenital (some sexually transmitted) and respiratory infections are included in the most frequent causes of morbidity at different age populations, mainly in developing countries. In this chapter, updates on the advances in the applications of probiotics in both fields are reviewed, and their mechanisms of action are suggested. The revision deals with in vitro and in vivo experimental models, including the small amount of clinical evidence of probiotic effects in humans. Some critical technological parameters in the design of foods or pharmaceutical products containing probiotic microorganisms are also discussed.*

### 8.1. Introduction

The historical application of probiotic microorganisms was related to the long and healthy life of Bulgarian inhabitants (Metchnikoff 1908). The application of this type of product was widely studied at the gastrointestinal (GI) tract, adminis-

tered in either foods or pharmaceuticals; however, new evidence of their application to different human and animal mucosae has been reported.

The definition of probiotics was almost exclusively referred to or related to the GI tract, and directed to the reestablishment of the GI microbiota. Probiotic was first defined by Lilley and Stillwell (1965), in opposition to antibiotics, as “substances secreted by one microorganism to stimulate the growth of other microorganisms.” Then, Fuller (1989) defined probiotic as “a live food supplement that affects the host beneficially improving the intestinal microbial balance,” but some years later, he redefined probiotic as “simple or mixed microbial cultures that are administered to the host to exert a beneficial effect on the indigenous microflora.” Due to the existing discrepancies in the scientific world, probiotics were later defined as “live microbes that when administered in adequate amounts confer a health benefit to the host” (FAO/WHO 2001). In 2002, the International Scientific Association for Probiotics and Prebiotics (ISAPP) was founded by researchers working in this area to discuss the principles and rules of probiotic products. Thus, the previous definition of probiotics was accepted and modified by ISAPP members as follows: “live microorganisms which, when administered in adequate amounts, exert a beneficial physiological effect in the host health” (Reid et al. 2003a).

Lactic acid bacteria (LAB) and related microorganisms historically used in the elaboration of



fermented foods are the prototype of probiotic microorganisms. LAB are also very active members of the indigenous microbiota of different mucosal areas of both poikilothermic and homeothermic animals, including humans. Nowadays, many probiotic products are available, some containing LAB originally present in foods and others containing probiotic bacteria isolated from the same organism tract to which they will be administered. Therefore, both types of microorganisms, allochthonous and autochthonous, can exert a beneficial effect on the host. The decision of using any of these two types of microorganisms will depend on their intrinsic colonization capability to maintain stable populations in the targeted area.

## 8.2. Urogenital Tract (UGT)

### 8.2.1. UGT Infections (UGTI)

UGTI constitute a public health problem affecting 1 billion people every year throughout the world and causing high health-care cost (Reid and Bruce 2006). UGTI can be classified into genital tract infections (GTI) and urinary tract infections (UTI). GTI are either sexually transmitted (STI) or non-sexually transmitted (non-STI). Each year, there are 340 million estimated new cases of curable STI as well as many millions of incurable viral STI, including 5 million Human Immunodeficiency Virus (HIV) infections (<http://www.who.int/reproductive-health/stis/index.htm>). The most common STI are trichomoniasis caused by *Trichomonas vaginalis*; chlamydiasis produced by *Chlamydia trachomatis*; gonorrhoea caused by *Neisseria gonorrhoeae*; syphilis produced by *Treponema pallidum*; chancroid caused by *Haemophilus ducreyi*; genital warts most frequently produced by types 6 and 11 Human Papilloma Virus (HPV); HIV caused by type 1 or 2 HIV; and genital herpes mainly caused by type 2 (HSV-2) herpes simplex virus. Among the non-STI the following syndromes are included: bacterial vaginosis (BV) of unknown etiology, but associated with the overgrowth of *Gardnerella vaginalis*, *Mycoplasma hominis*, *Peptostreptococcus* sp., *Bacteroides* sp., and other anaerobic bacteria; vul-

vovaginal candidiasis (VVC), produced mainly by *Candida albicans*; and aerobic vaginitis, with associated vaginal microbiota constituted by aerobic microorganisms, such as Group B *Streptococcus*, *Escherichia coli*, and *Staphylococcus aureus* (Donders et al. 2002).

In women, GTI may cause gynecological complications such as vaginal discharge, vaginosis, vaginitis, cervicitis, endometritis, genital ulcers, or pelvic inflammatory disease. Ongoing infection of the upper genital tract leads to infertility and ectopic pregnancies. On the other hand, infection during pregnancy may result in premature rupture of membranes and preterm delivery, and risk of neonatal infection and death.

On the other hand, UTI can mainly be caused by *E. coli*, *Staphylococcus saprophyticus*, *Enterococcus faecalis*, *Klebsiella pneumoniae*, and *Proteus mirabilis*. The origin of the uropathogens in uncomplicated UTI can be the fecal microbiota. UTI account for 8 million patient visits annually worldwide with a high rate of recurrences in adult women (Fihn 2003). In adults, these diseases may cause painful, frequent urination with a feeling of incomplete emptying of the bladder, perineal pain, fever, chills, and back pain. Most UTI are asymptomatic in elderly patients while symptoms are nonspecific in small children constituting high risk of renal failure.

Conventional UGTI (GTI and UTI) are treated mainly with antibacterial, antifungal, antiparasitic, or antiviral agents. However, frequent exposure to these substances can provoke the imbalance of the indigenous microbiota leading to the emergence of drug-resistant strains and adverse effects, high recurrence rates, and secondary infections. These therapies also represent high costs for the health-care systems in all countries. Thus, alternative therapeutic procedures such as the use of probiotic products offer alternative management regimens to antimicrobial treatments and their prophylaxis (Reid and Bruce 2003).

Currently, several probiotic products to restore vaginal microbiota exist in the international market, for example, vaginal tablets (Gynoflor®, Medinova, Zurich, Switzerland; Normogin, Laboratory Baldacci, Pisa, Italy), vaginal capsules (Döderlein



Med, Novartis Consumer Health GmbH, Muenchen, Germany; EcovagFlora™, Bifodan A/S, Hundested, Denmark; Fermalac® vaginal, Institut Rosell Inc., Montreal, Canada; and Lactonorm®, Geymonat, Anagni, Italy), vaginal suppositories (Lactinex®, Omega Laboratory, Buenos Aires, Argentina; Tropivag®, Finadiet, Buenos Aires, Argentina; and Vagiflor®, Asche AG, Hamburg, Germany), and oral capsules (Fem-Dophilus®, Jarrow Formulas, Los Angeles, CA). However, only few of these probiotic products have been properly proven in clinical studies and published in scientific databases; many of them being unreliable with regard to their content (microorganisms used and number of viable cells; Hughes and Hillier 1990; Reid and Bruce 2006).

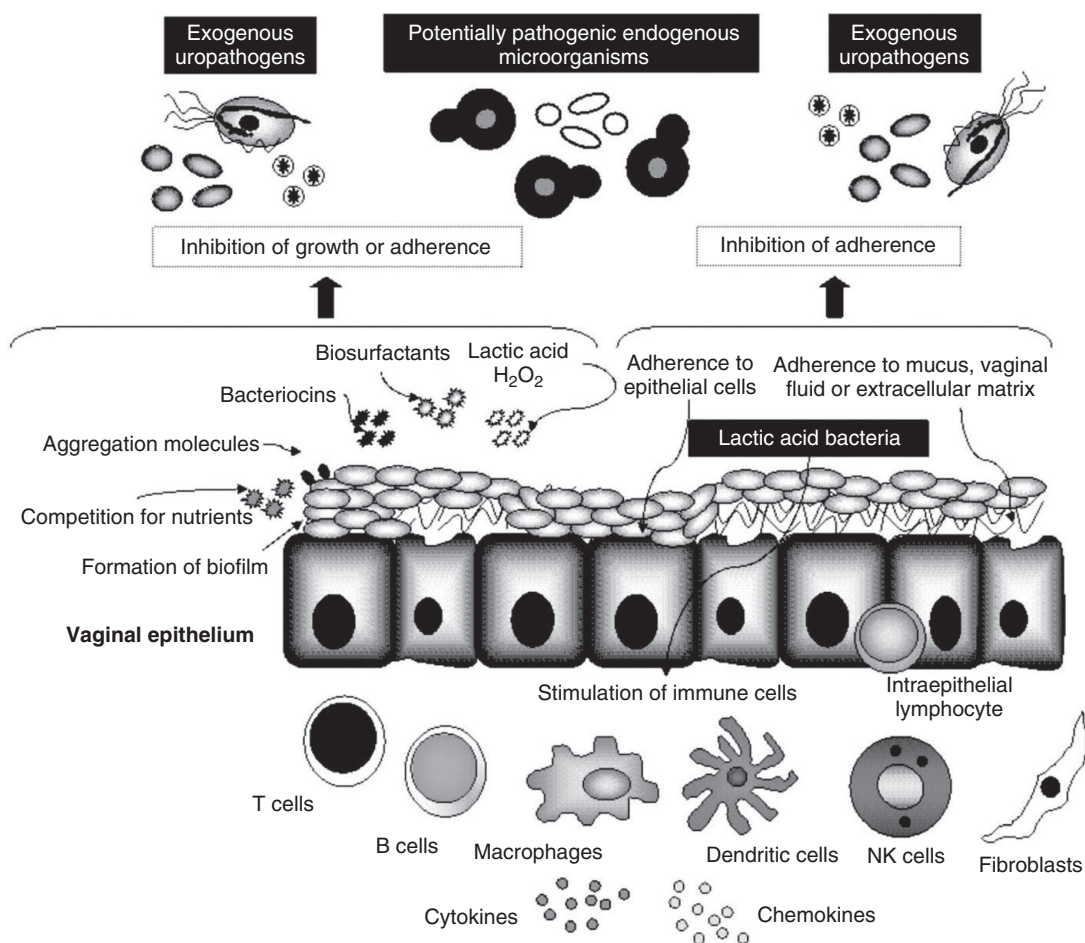
### 8.2.2. Selection of Probiotic Microorganisms: In Vitro Assays

The probiotic strains to be used in UGTI must belong to Generally Regarded as Safe microorganisms (e.g., *Lactobacillus*, *Pediococcus*, *Bifidobacterium*, and some *Streptococcus* strains). The correct taxonomic identification at the genus/species level by applying internationally accepted molecular tools is an outmost requirement for the use of probiotic microorganisms (Reid et al. 2003b). Selection of potentially probiotic strains can be performed with *in vitro* assays to evaluate functional and physiological characteristics thought to be beneficial for UGTI treatments. The proposed mechanism for probiotic LAB for UGT treatments is presented in Figure 8.1.

**Biofilm formation: Adhesion, aggregation, and production of biosurfactants.** As a first step for the colonization of the host, probiotic bacteria as well as indigenous microorganisms must be able to adhere to the epithelial cell surface, to the mucus covering the epithelial tissue, or to the connective or basal membrane constituents (collagen, fibronectin, laminin, and proteoglycans). Many nonspecific interactions may occur between bacterial and host surfaces such as electrostatic or hydrophobic interactions or H-bonds. Later, some nonspecific or specific adhesins, proteins, lipoteichoic acids, S-layers,

and polysaccharides of the LAB surface may be involved in the adhesion to host cells (Schär-Zammaretti and Ubbink 2003). Once bacteria are adhered to the host mucosa, they should form a protective biofilm in which bacterial behavior is completely different from that of planktonic cells (Maldonado et al. 2007). The phenomena related to the surface characteristics of the bacterial cell, such as adhesion, biosurfactant production, and auto- and co-aggregation capabilities could be involved in biofilm formation (Lepargneur and Rousseau 2002). Adherence or/and biofilm formation can prevent the income of pathogenic microorganisms, acting either by a competitive exclusion mechanism where specific interactions are involved or by steric hindrance, where nonspecific bonds are settled between bacteria and the host.

LAB strains showed the ability to inhibit the attachment of *S. aureus* and Group B streptococci to vaginal epithelial cells (VEC) but not the attachment of *E. coli* (Zárate and Nader-Macías 2006a). Spurbeck and Arvidson (2008) reported that vaginal lactobacilli reduced the *in vitro* adherence and invasion of *N. gonorrhoeae* to endometrial epithelial cells and displaced adherent gonococci by an unknown mechanism. Also, *Lactobacillus crispatus* CTV-05 was tightly adhered to VEC from women with or without recurrent UTI (Kwok et al. 2006). In addition, *in vitro* adhesion to VEC by vaginal lactobacilli was not affected by the pH value in the range of 4–7 (Ocaña and Nader-Macías 2001). Velraeds et al. (1998) reported that biosurfactants produced by lactobacilli inhibited the initial adhesion of some microorganisms responsible for UGTI (*Ent. faecalis*, *E. coli*, and *Staphylococcus epidermidis*) by steric exclusion. On the other hand, *Lactobacillus johnsonii* CRL1294 and *Lactobacillus salivarius* CRL1328 showed remarkable auto-aggregating patterns associated with a protein factor of the bacterial cellular surface (Ocaña and Nader-Macías 2002). The auto-aggregation ability of *Lact. johnsonii* CRL1294 increased mainly at low initial pH of the growth media, a remarkable characteristic for their survival in the acidic vaginal environment (Juárez Tomás et al. 2005). Also, co-aggregation of *Lact. johnsonii* CRL1294 and *Lact. salivarius*



**Figure 8.1.** Proposed mechanisms of probiotic LAB for UGT infections treatment. Once administered, LAB may form adherent biofilm, produce antagonistic substances, compete for nutrients, and stimulate the mucosal or systemic immune system to protect the host against pathogenic or potentially pathogenic microorganisms.

CRL1328 with vaginal *Candida* sp. was reported (Ocaña and Nader-Macías 2002).

**Production of inhibitory substances and enzymes, and nutrients availability.** Production of organic acids (mainly lactic acid), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), and bacteriocins is deemed to be a fundamental criterion for the selection of probiotic microorganisms. Lactobacilli have been recognized as the predominant microbiota of a healthy human vagina and are responsible for maintaining an acidic environment (pH < 4.5). Moreover, the predominance

of H<sub>2</sub>O<sub>2</sub>-producing LAB was related to a healthy vaginal tract (Cherpes et al. 2008).

Antagonistic substances produced by vaginal microorganisms were observed to inhibit the *in vitro* growth of different vaginal pathogens (Klebanoff and Coombs 1991; Okkers et al. 1999; McLean and Rosenstein 2000; Aroutcheva et al. 2001; St. Amant et al. 2002; Atassi et al. 2006; Todorov et al. 2007). In particular, strains of vaginal lactobacilli inhibiting the growth of *E. coli*, *S. aureus*, *Streptococcus agalactiae*, *Ent. faecalis*, *Klebsiella* sp., *N. gonorrhoeae*, and *G. vaginalis* by lactic acid production

were reported (Juárez Tomás et al. 2003a). In addition, the inhibition of *S. aureus* in mixed cultures was attributed to the production of H<sub>2</sub>O<sub>2</sub> by vaginal lactobacilli (Ocaña et al. 1999a; Juárez Tomás et al. 2003b).

Besides the food applications of bacteriocins in the last years, these substances were thought to be useful as preventive or therapeutic agents in the UGT. The supernatant of the salivaricin-producing *Lact. salivarius* CRL1328 inhibited the growth of *N. gonorrhoeae*, *Enterococcus faecium*, and *Ent. faecalis* (Ocaña et al. 1999b; Juárez Tomás et al. 2002a). The characteristics of this salivaricin (heat resistance and broad pH range of activity as well as its production in simple and low-cost growth media) favor its inclusion as an additive in probiotic products. Dover et al. (2007) demonstrated the safety of lactocin 160, an antimicrobial peptide produced by vaginal *Lactobacillus rhamnosus* 160, by employing a human tissue model of ectocervical cells.

Vaginal microbiota could also be modulated by nutrient competition. Once in the biofilm, the nutrients' availability for embedded microorganisms is limited as compared with that for planktonic cells, thus providing a selective advantage (Freter and Nader-Macías 1995). In particular, arginine has been demonstrated to be a growth-stimulating amino acid for anaerobic vaginal pathogens associated with BV. Therefore, a probiotic strain with arginine deiminase activity would decrease the arginine level in the vaginal environment inhibiting the growth of those pathogenic microorganisms (Famularo et al. 2001; Rousseau et al. 2005). The properties of beneficial microorganisms determined by applying *in vitro* assays must be verified through *in vivo* studies in both animal and clinical trials to guarantee the beneficial effects in the host. The suggested mechanisms of action for probiotics are probably the addition or synergy of different interactions between probiotic microorganisms and the host cells.

### 8.2.3. In Vivo Assays: Animal Models

The purpose of using colonization or infection animal models is to closely mimic the protective properties of the indigenous or probiotic microor-

ganisms in humans. However, because no animal model perfectly reflects human infection by pathogens or colonization by autochthonous strains, different models have been proposed. Dogs, mice, monkeys, pigs, as well as rabbits and rats, have been used to study UGTI (Johnson and Russell 1996). The urinary tract of mice and nonhuman primates contain digalactoside receptors similar to those present in the human urinary tract (Hagberg et al. 1983). When the preventive and therapeutic effects of lactobacilli in the UGT were evaluated using a mice model (Nader de Macías et al. 1992; Silva de Ruiz et al. 1993; Nader de Macías et al. 1996), lactobacilli increased the effectiveness of antimicrobial agents (e.g., norfloxacin) in the prevention and treatment of UTI caused by *E. coli* (Silva de Ruiz et al. 1996; Silva de Ruiz and Nader-Macías 2006). Furthermore, the use of estrogens combined with *Lactobacillus fermentum* CRL1058 decreased *E. coli* infection and increased *Lact. fermentum* colonization. No histological modifications were observed in these probiotic treatments (Silva de Ruiz et al. 2001, 2003, 2004) and both viable and nonviable *Lact. fermentum* cells can be used efficiently as probiotics in *E. coli* infections (Silva de Ruiz and Nader-Macías 2007). Likewise, Asahara et al. (2001) reported the antimicrobial activity of the probiotic *Lactobacillus casei* Shirota against *E. coli* in a murine UTI model, and Fraga et al. (2005) characterized an indigenous *Lactobacillus murinus* that showed preventive and therapeutic effects on *Proteus mirabilis* infection. Also, Winberg et al. (1993) reported that vaginal colonization in monkeys disturbed by the use of amoxicillin could be restored by flushing the vaginal microbiota obtained from healthy monkeys (Herthelius et al. 1989; Herthelius-Elman et al. 1992). Also, Patton et al. (2003) demonstrated that a single intravaginal application of *Lact. crispatus*-containing capsules resulted in vaginal colonization. However, negative results were obtained in estrogenized BALB/c mice challenged with *T. vaginalis* and treated with *Lact. acidophilus* (McGrory and Garber 1992).

Vaginal colonization of BALB/c mice by intravaginal inoculation with human lactobacilli was assayed. Most *Lactobacillus* strains were able to

stimulate the number of IgM-producing cells at different levels; however, stimulation of IgA-producing cells was strain-dependent. None of six strains assayed produced adverse effects (Vintiñi et al. 2004). In addition, *Lact. paracasei* CRL1289 demonstrated a mice-protective effect against *S. aureus* infections (Zárate et al. 2007).

The described *in vivo* assays indicate that adhesion, colonization, or immune system stimulation depend on specific characteristics of the bacterial strain. Thus, results obtained with one strain or experimental model cannot be extrapolated; however, they may be used as indicators for basic studies to further evaluate each particular situation.

#### 8.2.4. Clinical Assays

Several clinical studies have reported the application of probiotics in UGTI to artificially restore unbalanced microbiota. Different trials showed that the combination of *Lact. rhamnosus* GR-1 and *Lact. fermentum* RC-14 may reduce the risk of UTI and vaginal colonization of pathogenic microorganisms and improve the maintenance of normal microbiota (Reid and Burton 2002; Reid et al. 2003c). Oral administration of these strains showed intestine and vagina colonization (Marelli et al. 2004), providing a major step in self-administration therapies. Strains RC-14 and GR-1 were able to colonize more effectively the vaginal tract than *Lact. rhamnosus* GG (Gardiner et al. 2002) highlighting the importance of proper selection of strains for urogenital probiotic applications. Moreover, the use of these lactobacilli together with antibiotic demonstrated to be effective in the eradication of BV in black African women (Anukam et al. 2006).

Other bacterial strains were evaluated as potential urogenital probiotics. The administration of vaginal suppositories containing *Lact. crispatus* GAI98332 seemed to be a safe and promising treatment for the prevention of recurrent UTI (Uehara et al. 2006). Similarly, Czaja et al. (2007) found that *Lact. crispatus* CTV-05 can be given as a vaginal suppository to healthy women with a history of recurrent UTI, with minimal side effects. On the

other hand, tampons loaded with a combination of *Lact. fermentum*, *Lact. casei* var. *rhamnosus*, and *Lactobacillus gasseri* did not improve the cure rate compared with placebo tampons (Eriksson et al. 2005).

Concerns about product stability and the limited documentation of strain-specific effects of some preparations used in UGTI control prevent recommendations for the use of *Lactobacillus*-containing probiotics in BV treatments. Several studies on the use of lactobacilli as prophylaxis of UTI remain inconclusive as a result of small sample sizes and use of invalidated dosing strategies (Falagas et al. 2007; Barrons and Tassone 2008). Although results on therapeutic effects of urogenital probiotics are not definitive, selection of scientifically based strains could provide a reliable alternative and preventive treatment to antibiotics in the future.

#### 8.2.5. Technological Parameters for Vaginal Probiotics

Determination of optimal conditions to obtain a high amount of viable cells of probiotic strains as well as the best storage conditions for the stability of selected microorganisms is a fundamental aspect in the design of probiotic products. To date, few studies describing the production processes of probiotic vaginal formulations have been published. The effect of physicochemical factors (e.g., growth medium, initial pH, incubation temperature, and oxygen tension) on the growth of probiotic vaginal strains were reported by Juárez Tomás et al. (2002b, 2004). The biomass of probiotic strains produced under optimum growth conditions were stored as concentrated cultures and successfully incorporated into the final product. The presence of sucrose or lactose and ascorbic acid used as lyoprotectors and pharmaceutical excipients, respectively, improved the recovery of viable cells and the expression of beneficial properties of vaginal lactobacilli during freeze-drying and long-term storage (Zárate and Nader-Macías 2006b; Juárez Tomás et al. 2009). However, the microorganisms' ability to adhere to

VEC or to produce lactic acid,  $H_2O_2$ , and bacteriocins was affected in different extents by the lyophilization conditions (Zárate and Nader-Macías 2006b; Juárez Tomás et al. 2009).

#### 8.2.6. Immune System at the UGT

The immune system represents an important component of the reproductive tract (Wira et al. 2005a; Witkin et al. 2007; Barrons and Tassone 2008; Ochiel et al. 2008) influencing many of the biological functions of the host. The regulation of the immune response of the UGT is very complex because this mucosal surface must deal with members of the indigenous microbiota, many potential pathogens, spermatozoa, and the fetus. Failure of the immune system to either eliminate pathogens in the reproductive tract or resist the attack of allogeneic sperm and fetus significantly compromises procreation as well as the mother's health while failures in the urinary tract immune system compromise women's well-being by recurrences and risks of renal infections.

The concept that the fetus is sterile and comes in contact with the first microorganisms while passing through the vaginal canal at birth supports the hypothesis that the acquired microbiota during and immediately after birth is necessary for the maturation of the newborn systemic and mucosal immunity, a concept that is being demonstrated nowadays at the intestinal tract. Recent studies, however, have shown that even though lactobacilli and bifidobacteria cells were not detectable in placenta (vaginal or caesarean obtained) by cultivation, their DNA was detected in most of them (Satokari et al. 2008). These results suggested that horizontal gene transfer of bacterial DNA from mother to fetus may have occurred via the placenta. It is well known that specific unmethylated CpG oligonucleotide motifs present in DNA are able to activate Toll-like receptor-9 (TLR-9) and trigger Th1 (helper T cells) type immune response. Although the newborn infant is considered immunologically immature, the exposure to bacterial DNA may program the infant's immune development during fetal life earlier than previously considered.

The immune system of adult women, which includes a complex array of cells and molecules that interact to provide protection to the host, has been described in detail only in the last years (Wira et al. 2005a). The central lymphoid organs contribute to the ontogenesis of the different immune cells while the peripheral lymphoid organs, in which the mucosal lymphoid tissue is included, contribute to the orchestration of the immune response. Most of the antigens penetrate the body through the mucosa so the mucosal immune system plays a key role in the defense response to pathogens. The UGT mucosal system of the urinary and reproductive tracts is part of the common mucosal immune system, developed and closely connected with the gastrointestinal, respiratory, and glandular systems. The existence of this common immune system through all the mucosal tracts has modified the conception of the way or route of administration of a microbial supplement, to exert its *in situ* or at-very-distant-place effect. This subject is being deeply studied in relationship to the "tolerance" phenomenon and to broad areas of research at the GI tract.

The mucosal epithelium of the UGT provides the first barrier of defense of specialized organs, and is equipped to respond to pathogens while sustaining commensal microorganisms. This epithelium also participates in the complicated set of networks and interactions that are boosted once probiotic microorganisms or their antigens interact with it. These UGT mucosal surfaces offer a wide area for the interaction of antigens with the immune system and include places for antigenic exclusion, antigenic samples, and immune regulation (briefly shown in Fig. 8.1).

The UGT mucosal immune system and the systemic immune system are similar in the protection against potential pathogens, which is provided by innate (first line of defense) and adaptive immunity (Wira et al. 2005a, 2005b; Witkin et al. 2007; Barrons and Tassone 2008; Ochiel et al. 2008). An effective specific immune response against pathogens requires the antigen process by the Antigen-Presenting Cells, and the protection is achieved through humoral immunity (specific antibodies produced by B cells)



or cell-mediated immunity (direct or indirect destruction of pathogens by T cells).

When studying the microbial composition of the UGT and the susceptibility to UGTI, the genetic polymorphism of each person, which varies among the different ethnic groups, should be considered. This individual genetic capacity is defined as small changes in the DNA sequence of a gene occurring between individuals that influence the production of high or low levels of anti- or pro-microbial factors. Polymorphism in several genes (Interleukin-1B [IL-1B], Tumor Necrosis Factor- $\alpha$  [TNF- $\alpha$ ], TLR-2, TLR-4, etc.) may influence the predisposition to BV, VVC, UTI, and risk for preterm deliveries in different ethnic groups (e.g., Caucasian, black, and Brazilian women; Cauci et al. 2007; Genc et al. 2007; Giraldo et al. 2007; Karoly et al. 2007; Tabel et al. 2007). These examples indicate the complexity of the microbial–host interactions, in which the relationships established between the probiotic bacteria and the host immune system are included. A few references show the effects of LAB on the vaginal immune system (Table 8.1). These studies demonstrated an inverse relationship between vaginal LAB

levels (mainly lactobacilli) and IL-1B, IL-6 and IL-8, which are increased in several UGTI.

Regarding the urinary tract, IL-6 levels were significantly elevated in urine with *E. coli* higher than  $10^5$ , concentration corresponding to a level of significant bacteriuria and useful to detect the UTI infections. Rodríguez et al. (2008) reported that a value  $>15$  pg/ml of IL-6 is a strong indicator of acute pyelonephritis in children. No studies have related the number of lactobacilli with the levels of these types of mediators in urine yet.

Most recent research in this area aims to study the relationship between the stability and susceptibility of the UGT and RT microbiota to infections with the genetic makeup of each person.

#### 8.2.7. LAB as Vaccine Vectors for the UGT

Vaccination is one of the most effective and nonexpensive ways of preventing or treating diseases. Some of the highest challenges in the area are that vaccines should be able to elicit a strong and durable immune response. The mucosal administration of vaccines may elicit a wide range of immune

**Table 8.1.** Effects of LAB on the vaginal immune system.

Type of study	Status of patients included	Results	Reference
Epidemiological study	Pregnant women	Absence of lactobacilli associated with an increased cervical IL-8 and a higher risk of preterm labor	Sakai et al. (2004)
	Pregnant women with or without BV	Inverse relationship between the number of LAB (lactobacilli and <i>Weissella</i> ) and IL-1B levels	Hedges et al. (2006)
	Women with or without BV	Inverse correlation between IL-6 and IL-8 levels and <i>Lact. crispatus</i> , but direct relation regarding <i>Atopobium vaginae</i> and <i>Gardnerella vaginalis</i>	Libby et al. (2008)
	HIV infected women	Inverse correlation between IL-8 and lactobacilli; direct correlation between IL-8 and <i>Candida</i>	Spear et al. (2008)
Exogenous administration of lactobacilli to women	Women with or without BV and VVC	Induction of synthesis of IL-8 mRNA and $\beta$ -defensin 2mRNA in human vaginal epithelia containing dendritic cells, after treatment with <i>Lact. jensenii</i>	Valore et al. (2006)



responses at different mucosal sites. Even though oral, intradermic, and subcutaneous vaccine administration have been highly efficient in eradicating dangerous infections in specific world regions or countries, new strategies are now sought for vaccine delivery at various mucosal sites. The inoculation by the intranasal, intravaginal, or intracolonic route proves to be an excellent way to protect against sexually and vertically transmitted diseases (McConnell et al. 2008).

A new generation of vaccines is being developed using specific LAB strains and some probiotic Gram (+) lactobacilli. LAB as delivery vehicles have been mainly focused on the construction of mucosal vaccines, with efforts being devoted to the generation of genetic tools for antigen expression in different bacterial locations. The use of bacteria as vehicles for vaccine delivery implies construction of recombinant strains that contain the gene cassette encoding the antigen of interest. For example, Grangette et al. (2004) showed a stronger specific response on vagina against a model of the C subunit of tetanus toxin coupled to a cell wall mutant of *Lactobacillus plantarum*. For contraceptive purposes, Yao et al. (2006, 2007) have coupled the human chorionic gonadotrophin- $\beta$  (hCG- $\beta$ ) gene into live lactobacilli. Among the different inoculation routes assayed, the vaginal mucosal appears to be a better immunization pathway to induce higher anti-hCG- $\beta$  antibody levels in the reproductive tract. On the other hand, a strategy for preventing HIV was presented by Chang et al. (2003) in engineering the commensal bacterium *Lact. casei* to secrete two domains CD4 proteins that bind HIV type 1 gp 120 and inhibit the entry of the virus into the host target cell. Several authors have proposed that recombinant LAB might exert a control of genital cancer produced by HPV (Cortes-Perez et al. 2005, 2007; Poo et al. 2006). Cortes-Perez et al. (2005, 2007) have shown that the coadministration of recombinant lactococci and *Lact. plantarum* displaying HPV antigens at their surface and secreting IL-12 displayed therapeutic effect on HPV-16 induced tumor in mice by induction of antitumor-specific systemic and mucosal responses. Similarly, HPV-16

E7 antigen displayed on *Lact. casei* induces E7-specific antitumor effects in C57/BL6 mice (Poo et al. 2006).

### 8.3. Respiratory Tract (RT) and Probiotics

The UGT and the respiratory system are part of the mucosal immune syndrome; however, the mechanisms involved in the protection of infections at both levels are different and will be considered as separate items in the present review.

#### 8.3.1. Incidence and Relevance of RT Infections (RTI)

The RT is an important entry route of pathogenic microorganisms, which begin their replication in this area before spreading to the rest of the body. Acute lower RTI constitute a persistent and pervasive public health problem. They cause a greater burden of disease worldwide than HIV infection, malaria, cancer, or heart attacks (Mizgerd 2008). The relative importance of the different causes of mortality in children aged less than 5 years varies across world regions; pneumonia remains the major disease everywhere (Rudan et al. 2008). The incidence of childhood pneumonia is estimated to be 0.29 episodes per child-year in developing countries and 0.05 episodes per child-year in developed countries. This rate means about 156 million new episodes each year worldwide, from which 130 million occur in the developing world (Rudan et al. 2008). The leading bacterial cause is *Streptococcus pneumoniae*, being identified in 30%–50% of pneumonia cases; the second most common organism isolated is *Haemophilus influenzae* type b (Hib; 10%–30% of cases), followed by *S. aureus* and *K. pneumoniae* (Rudan et al. 2008). In the United States, these bacteria cause more disease and death than any other infection; little change in mortality due to RTI has occurred for 50 years. With the exception of oxygen therapy for severe pneumonia and zinc supplementation to prevent disease, little clinical amelioration of pneumonia has been achieved in the last 20 years

(Scott 2008). Researchers of the Board of Science and Technology for International Development believed that knowledge on the modulating effects of nutritional status and immune deficiency on the inflammatory response would offer practical opportunities to influence disease outcome. Thus, it is necessary to have effective preventive methods to improve the defenses of the host against the challenge of pathogens, particularly relevant in immunocompromised hosts.

LAB have been used for the development of probiotic foods with the ability to stimulate the immune system, which would increase resistance to infections even in immunocompromised hosts (Cano and Perdígón 2003; Jain et al. 2008; Chen et al. 2009; Corr et al. 2009; Jandu et al. 2009). On the other hand, the advances in the molecular biology of LAB have enabled the development of recombinant strains expressing antigens from various pathogens that have been proven effective in inducing protective immunity. Hence, probiotics represent an important alternative to reinforce the host defenses against respiratory infections.

### 8.3.2. Experimental Model Studies

*Respiratory infections in immunocompetent hosts.* Although most research concerning probiotic-mediated enhancement of the immune response was focused on GI tract pathogens, few recent studies emphasize whether probiotics might sufficiently stimulate the common mucosal immune system to provide protection to other mucosal sites as well (Cross 2002). In this sense, Yasui et al. (1999) focused their investigations on the potential effect of LAB in the improvement of immunity against influenza virus infection. The authors found that virus-vaccinated mice previously fed *Bifidobacterium breve* strain YIT4064 showed markedly increased titers of serum anti-influenza IgG antibodies and higher survival rate against intranasal influenza challenge compared with control-fed/virus-vaccinated mice. The same authors reported that oral administration of *Lact. casei* strain Shirota to aged mice activates the cellular immune system and reduces influenza virus titer in the upper

RT (Hori et al. 2002). Thus, treated aged mice showed improved protection against the viral infection. In addition, the authors reported later that neonatal and infant mice treated orally with the same *Lact. casei* strain showed enhanced pulmonary natural killer cell activity and IL-12 production that improved resistance against upper RT influenza infection (Yasui et al. 2004).

The potential preventive and therapeutic effects of probiotics in the immune response against respiratory pathogens were evaluated. *Lact. casei* CRL431, a well-documented probiotic strain (Perdígón et al. 2001; Gauffin Cano et al. 2002; Villena et al. 2005; Agüero et al. 2006; Racedo et al. 2006), and a simulated commercial yogurt showed enhanced protection against the aerosolized challenge with *Pseudomonas aeruginosa* using an experimental model with young mice (Alvarez et al. 2001). The pathogen clearance rate from the lungs was markedly increased in treated yogurt-fed mice; an upregulation of the phagocytic capacity of alveolar macrophages as well as an increase in total serum and bronchoalveolar fluid IgA and IgM levels was observed. Also, the improvement in the immune response of adult immunocompetent mice challenged with *Strep. pneumoniae* showed that the immunostimulating properties of LAB were strain- and dose-dependent (Alvarez et al. 2001; Cangemi de Gutierrez et al. 2001; Villena et al. 2005, 2006; Racedo et al. 2006; Salva et al. 2008). Proper-dose oral administration of *Lact. casei* CRL431, *Lactococcus lactis* NZ9000, a strain used for the expression of heterologous proteins (Le Loir et al. 2005), as well as the probiotic strain *Lact. casei* CRL1505 isolated from goat milk, improved *Strep. pneumoniae* clearance rates in the lungs and blood; it also improved the survival of infected mice and reduced lung injuries (Racedo et al. 2006; Salva et al. 2008, personal communication; Villena et al. 2008).

*Respiratory infections in immunocompromised hosts.* The effect of *Lact. casei* CRL431 or probiotic yogurt on the recovery of the innate and adaptive immune responses against *Strep. pneumoniae* RTI was determined in a protein-malnourished mice

model (Villena et al. 2005). *Lact. casei* or yogurt administration during a repletion diet accelerated the normalization in the number and function of phagocytes (Villena et al. 2005, 2006). In addition, an improvement of the antibody response to pneumococcal infection, probably mediated by different cytokine profiles induced by *Lact. casei*, was detected (Agüero et al. 2006; Salva et al. 2008). An early normalization of the local and systemic humoral immune responses against pneumococci in malnourished mice repleted with balanced conventional diet; supplemental probiotic yogurt was also observed (Villena et al. 2006). These results suggest that immunoenhancing probiotic LAB accelerate the recovery of the humoral immune response against respiratory pathogens in malnourished hosts.

### 8.3.3. Clinical Studies

Few studies using human models have been assessed to establish the potential effect of probiotics on the improvement of respiratory defences. Hatakka et al. (2001) conducted a randomized study in which children consumed milk supplemented with *Lact. rhamnosus* GG or placebo unsupplemented milk over a period of 7 months. Children who consumed *Lact. rhamnosus* GG reported a significantly lower incidence of RTI and a trend toward less frequent antibiotic treatments for respiratory complications compared with the control group. Another controlled pilot study evaluated the effect of 3 weeks supplementation with milk fermented with yogurt cultures and *Lact. casei* DN-114 001 on the incidence and severity of gastrointestinal and respiratory winter infections in elderly people (Turchet et al. 2003). Duration of pathologies was significantly reduced in the probiotic-treated group compared with the controls. Regular intake of probiotics may reduce potentially pathogenic bacteria in the upper RT (Glück and Gebbers 2003) indicating a linkage of the lymphoid tissue between the gut and the upper RT. The incidence and the severity of symptoms in common cold infections were significantly diminished in the probiotic group using a randomized double-blind placebo-controlled intervention study (Winkler et al. 2005). This effect was attributed to

the high levels of leukocytes, lymphocytes, CD4+ and CD8+ T –cells, and monocytes found in the probiotic group. The duration of common cold episodes and fever was shorter in the probiotic-treated group using *Lact. gasseri* PA 16/8, *Bifidobacterium longum* SP 07/3, and *Bifidobacterium bifidum* MF 20/5 for 3 months than in the control group, which was also correlated with an enhanced cellular immunity (de Vrese et al. 2005). Moreover, Cobo Sanz et al. (2006) found a statistically significant difference in favor of *Lact. casei* DN-114 001 fermented milk with respect to the duration of low RTI, bronchitis, and pneumonia. This study also reported a lower incidence of low RTI in children after the continuous consumption of *Lact. casei* DN-114 001 fermented milk as compared with those in the placebo group.

Recently, interesting studies were conducted on the effect of probiotics in the susceptibility to respiratory infections in athletes, bearing in mind that heavy exercise is associated with an increased risk of upper RTI. Marathon runners that received *Lact. rhamnosus* GG or placebo for a 3-month training period did not show significant differences in the incidence of respiratory infections between groups (Kekkonen et al. 2007). On the contrary, the administration of *Lact. fermentum* VRI-003, during a 4-month period of winter training, was associated with a substantial reduction in the number of days and severity of respiratory illness in a cohort of highly trained distance runners (Cox et al. 2008). Thus, most of the clinical studies reviewed support the use of probiotic LAB for the prevention of respiratory infections.

### 8.3.4. Respiratory Pathogens and LAB as a Delivery System of Antigens in Live Vaccines

Over the past decade, there has been increasing interest in the use of LAB as mucosal vaccines. Promising advances have been made in the design of both more efficient mucosal adjuvants and vaccine delivery systems using these microorganisms. With regard to protection against respiratory pathogens, only recombinant LAB expressing antigens of

*Strep. pneumoniae*, Respiratory Syncytial Virus, and severe acute respiratory syndrome (SARS)-Associated Coronavirus, have been reported (Falcone et al. 2006; Lee et al. 2006).

Strategies for the development of new vaccines against *Strep. pneumoniae* infections try to overcome problems such as serotype coverage, effectiveness in risk groups, and high costs, which are present in current available vaccines. Vaccines based on protein candidates that can induce protection in animal models are being evaluated. At present, recombinant strains expressing the pneumococcal surface protein A (PspA), the pneumococcal surface antigen A (PsaA), and the pneumococcal protective protein A (PppA) were obtained. Oliveira et al. (2006) expressed *Strep. pneumoniae* Psa A in different species of LAB such as *L. lactis*, *Lact. casei*, *Lact. plantarum*, and *Lact. helveticus*. Immunization with *L. lactis* induced very low levels of IgA (in saliva, nasal, and bronchial washes) and IgG (serum), probably due to the low amount of PsaA expressed in this strain. In contrast, *Lact. plantarum* and *Lact. helveticus* induced the highest antibody specific response (IgA and IgG). Moreover, only vaccination with recombinant lactobacilli but not lactococci led to a decrease in *Strep. pneumoniae* recovery from nasal mucosa upon a colonization challenge. These results indicate that intrinsic properties of each strain should be considered in the selection of LAB to be used as vectors for vaccines.

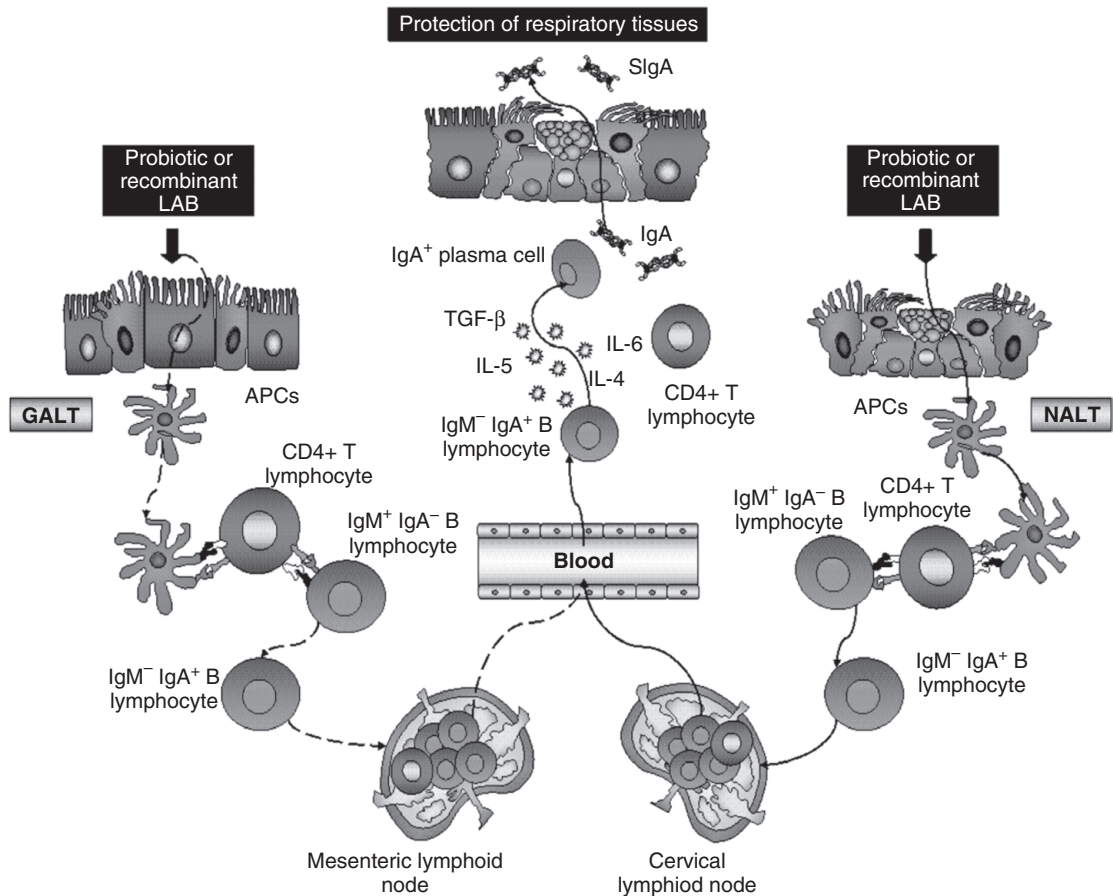
PspA, another pneumococcal protein, plays an important role during systemic infection through the inhibition of complement deposition on the pneumococcal surface. Hanniffy et al. (2007) successfully evaluated *L. lactis* intracellularly producing PspA as an intranasal mucosal vaccine against pneumococcal disease in a mice model, the effect being associated with a shift toward a Th1-mediated immune response. In a recent work (Campos et al. 2008) the N-terminal region of clade 1 PspA was constitutively expressed in *Lact. casei*. Nasal immunization with recombinant strain (*Lact. casei*-PspA 1) induced anti-PspA antibodies able to bind to pneumococcal strains carrying both clade 1 and clade 2 PspAs and to induce complement deposition

on the surface of the bacteria. After a systemic challenge with a virulent pneumococcal strain, an increase in survival of immunized mice was observed. This report represents an important contribution in the understanding of the mechanisms induced by recombinant strains.

The intrinsic immunostimulating properties of nasal administration of *L. lactis* NZ9000 was evaluated in a pneumococcal infection model. In appropriated dose, nasal administration increased the clearance rate of *Strep. pneumoniae* from the lungs and prevented the dissemination of pneumococci into the blood, an effect that correlates with upregulation of the innate and specific immune responses in both local and systemic compartments (Medina et al. 2008a). To improve the protection of the host against pneumococcal infection, the pneumococcal protein PppA was expressed in the strain NZ9000, and its efficacy to induce local and systemic immune responses in young and adult mice was assessed (Medina et al. 2008b). The results indicated that nasal vaccine with PppA expressed as a protein anchored to the cell wall of *L. lactis* elicited cross-protective immunity against different pneumococcal serotypes, afforded protection against both systemic and respiratory challenges, and induced protective immunity in mice of different ages (Medina et al. 2008b).

Lee et al. (2006) developed a novel surface antigen display system for LAB. The Spike (S) protein segments of SARS-associated coronavirus were expressed in the surface of *Lact. casei*. Oral and nasal inoculations to mice with recombinant *Lact. casei* induced high levels of serum IgG and mucosal IgA against S peptides that neutralized the SARS pseudoviruses; oral immunization showing higher levels of neutralizing-antibody response than nasal administration. On the basis of previous findings, a potential mechanism to explain the influence of LAB on the systemic and respiratory immune response is shown in Figure 8.2.

Beyond the advances described in the development of live LAB vaccines protecting against respiratory pathogens, gaps in the understanding of the full potential of recombinant LAB remain unanswered. Further studies are necessary for a



**Figure 8.2.** Systemic and respiratory immune response induced by LAB. Probiotic or recombinant LAB are internalized by M cell in the gut (GALT) or nasal associated lymphoid tissues (NALT) after oral or nasal administration, respectively. The bacteria or their fragments interact with antigen presenting cells (APCs) that are activated to produce cytokines. Immature APCs migrate to the adjacent interfollicular T cell zone where they finish their maturation. These mature APCs process and present antigens to T cells. Activated CD4+ T cells induce proliferation and switching of IgM+IgA- B-cells that result in the generation of IgM-IgA+ B-cells. Then, the post-switch IgA-committed B-cells migrate to mucosal effectors sites where these antigen-sensitized IgM-IgA+ B-cells undergo terminal differentiation to IgA-producing plasma cells. Oral administration of LAB can induce mobilization of IgA-producing plasma cells to the respiratory tract and improve resistance against respiratory pathogens. However, local stimulation of LAB is more effective in improving respiratory and systemic immunity against pathogens.

better insight of the mechanisms involved in the mucosal protection, emphasizing the difficult and challenging task of combining vaccine vectors and adjuvants.

#### 8.4. Conclusions and Perspectives

Infections of the UGT and RT are related to high rates of morbidity and mortality. Probiotic applica-

tions in these tracts could help to improve life quality of risk and infected populations. However, several potential research areas remain to be addressed: (1) origin, doses, and period of treatments of probiotic strains required for each particular infection and tract; (2) studies of the immune response, identification of effector and regulatory molecules and immune cells involved; (3) nature of probiotic interactions with epithelial and immune



cells; and (4) relationship between stability of UGT and RT microbiota and their susceptibility to infection. More clinical and scientific evidence is needed to deeply understand the mechanisms by which probiotics exert beneficial effects in the UGT and RT.

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## Chapter 9

# Lactic Acid Bacteria as Live Vectors: Heterologous Protein Production and Delivery Systems

Anderson Miyoshi, Luis G. Bermúdez-Humarán,  
Marcela Santiago Pacheco de Azevedo, Philippe Langella, and Vasco Azevedo

*Lactic acid bacteria (LAB), widely used in the food industry, are present in the intestine of most animals, including humans. The potential use of these bacteria as live vehicles for the production and delivery of heterologous proteins of vaccinal, medical, or technological interest has therefore been extensively investigated. Lactococcus lactis, a LAB species, is a potential candidate for the production of biologically useful proteins. Several delivery systems have been developed to target heterologous proteins to a specific cell location (i.e., cytoplasm, cell wall, or extracellular medium). A promising application of L. lactis is its use as an antigen delivery vehicle for the development of live mucosal vaccines. The expression of heterologous proteins and antigens as well as the various delivery systems developed in L. lactis and its use as an oral vaccine carrier are discussed.*

### 9.1. Introduction

Lactic acid bacteria (LAB), ingested daily by humans, are widely used in the food industry for the production and the preservation of fermented products. LAB include a large number of Gram (+) cocci or bacilli belonging to a phylogenetically heterogeneous group. Their traditional use in the food industry confirms their lack of pathogenicity; they are Generally Recognized as Safe (GRAS) organisms.

Since the 1980s, many efforts have been made to better understand the molecular basis of LAB's

technological properties and to obtain better control of industrial processes involving LAB. This knowledge has led scientists to investigate their potential use in new applications, such as the production of heterologous proteins in bioreactors, in fermented food products, or directly in the digestive tract of humans and other animals. Some LAB, used as probiotic strains, naturally exert a positive action in lactose-intolerant consumers by providing lactase in the gut (de Vrese et al. 2001). Besides such natural benefits, another innovative application of LAB is the delivery of digestive enzymes to supplement pancreatic deficiency in humans. Some recombinant strains producing lipase have already been used with success in animals (Drouault et al. 2000, 2002). A new application for LAB, and probably the most promising, is their use as live delivery vectors for antigenic or therapeutic protein delivery to mucosal surfaces. Such engineered LAB are able to elicit both mucosal and systemic immune responses. Several research projects have examined *Lactobacillus* sp. and *Lactococcus* sp. as vectors. Efficient expression systems have already been developed for controlled and targeted production of the desired antigen to be presented to the gastrointestinal mucosal immune system (Wells et al. 1993b; Norton et al. 1995; Le Loir et al. 2001; Seegers 2002).

In this chapter, we describe advances concerning the use of LAB as live delivery vectors, focusing on *Lactococcus lactis*, the LAB model. Various

molecular tools have been developed to efficiently express antigens and therapeutic molecules at different cellular localizations. We report here on the systems developed to use *L. lactis* as a live vaccine and the effects of such an antigen presentation mode on the mucosal immune system.

## 9.2. LAB: The Context Behind Their Use as Live Vectors

LAB constitute a very heterogeneous group of microorganisms that occupy a wide range of ecological niches, from plant surfaces to animal guts. Although they are quite diverse, the members of this group have various characteristics in common as they are Gram (+), facultative anaerobes, non-spore forming, nonmotile, and principally possessing the capacity to convert sugars mainly into lactic acid, which led to the name group “lactic acid bacteria.” Currently, the LAB group includes species of the genera *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus*, and *Weissella*, which have less than 50% G+C content in their DNA (Stiles and Holzapfel 1997).

### 9.2.1. Industrial Importance of LAB

LAB, with few exceptions, obtain their energy from the conversion of sugars, mainly glucose, into lactic acid (homofermentative or homolactic routes) and/or lactic acid and other products (heterofermentative or mixed routes) (Carr et al. 2002). Consequently, LAB are, generally speaking, associated with the preparation of fermented foods, such as yogurt, cheese, acid milk, bread, butter, wine, sausages, pickles, and silage. This process, known as “lactic fermentation of foods,” goes back to about 8000 B.C. and constitutes one of the most ancient forms of food preservation used by humans (Tailliez 2001). Food preservation is not only a consequence of acidifying the medium (pH 4.5–3.5), but is also due to the production of numerous antibacterial agents, such as bacteriocins and organic compounds (van de Guchte et al. 2001). These two factors

inhibit the growth of an undesirable microbiota and/or are responsible for the development of desirable organoleptic properties, such as texture, aroma, and flavor of the final product.

### 9.2.2. LAB in Health and Nutrition

Among the various properties attributed to LAB, perhaps the most ancient and controversial is their capacity to promote beneficial effects, known as “probiotic” properties, for human and animal health (Fuller and Gibson 1997). Various species of LAB are claimed to act as probiotics, such as *Lactobacillus casei*, *Lactobacillus delbrueckii*, *Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Lactobacillus fermentum*, and *Lactobacillus reuteri*, and stand out for their therapeutic applications in the treatment and prevention of various disorders (Ouweland et al. 2002).

Historically, the first application involved the ability to digest lactose. This sugar, abundant in milk products, is not tolerated by many people who have congenital lactase deficiency. Clinical manifestations include diarrhea, abdominal colic, and flatulence. Curiously, these symptoms appear with milk ingestion, but are practically absent when yogurt is ingested. There are three explanations for this phenomenon: (1) live bacteria present in yogurt (*Streptococcus thermophilus* and *Lactobacillus bulgaricus*) consume the lactose during yogurt production; (2) live bacteria present in yogurt provide the deficient host with lactase supplement; and/or (3) they stimulate the endogenous production of this enzyme by the intestinal mucosa of the host (Roberfroid 2000).

A second application of LAB probiotics would be protection against pathogenic microbes. In this case, LAB function as a barrier, impeding the colonization of the gastrointestinal tract by pathogenic bacteria. The most remarkable results have been obtained with some types of lactobacilli and bifidobacteria, which have been found to be particularly effective in treating diarrhea in newborns (Ouweland et al. 2002). Another well-established application is the stimulation of the immune system of the host. Among lactobacilli, *Lact. casei* has been found to

be capable of stimulating an immune response in children who take an oral vaccine against rotavirus; this virus causes acute diarrhea in infants and young children in developing countries (Ouweland et al. 2002).

Although some of these therapeutic applications have been questioned, growing concerns about health and well-being, along with an interest in consuming natural foods, have drawn considerable attention to probiotics, especially in the dairy products industry. Currently, many milk products containing probiotics are available on the market, fermented milk being the most common. There is conclusive evidence that consuming live LAB induces probiotic effects. However, more information about their modes and mechanisms of action are needed before they can be considered for the prevention and treatment of diseases.

### 9.3. New and Future Uses of LAB

At least three types of “nonfood” use are envisaged for LAB: (1) production in fermentors of economically important proteins, (2) production of foods containing biotechnological proteins, and (3) construction of live vaccines.

#### 9.3.1. Production of Proteins in Fermentors

A classic application consists of the use of LAB to produce economically important molecules in fermentors. For this purpose, the secretion of molecules would be advantageous compared with intracellular production to achieve an easy purification of the final product from a continuous cell culture, avoiding the formation of intracellular protein aggregates (Langella and Le Loir 1999).

#### 9.3.2. Production of Proteins within Foods

Another application of LAB would be the production of enzymes within foods in order to (1) modify the organoleptic properties of the products, (2) prevent the growth of undesirable microbiota, (3) accelerate cheese maturation, (4) optimize silage production, and (5) overcome individual digestive enzyme defi-

ciencies, such as the lack of lipases. In this last case, supplementation with bacterial lipase could be a viable alternative. The efficacy of providing these enzymes via bacteria has been demonstrated in dogs (Suzuki et al. 1997) and pigs (Drouault et al. 2002) with experimentally induced steatorrhea. In this latter case, a recombinant *L. lactis* strain expressing the *lip* gene (lipase) of *Staphylococcus hyicus* (Drouault et al. 2000) was administered to pigs with pancreatic insufficiency, resulting in a 15% increase in the capacity to absorb lipids.

#### 9.3.3. Construction of Live Vaccines

The use of live microorganisms as cellular vehicles for the production and presentation of antigens has contributed significantly to the development of new vaccines. Currently, most of these vehicles are derived from pathogenic microorganisms, such as *Salmonella typhimurium*, *Yersinia enterocolitica*, *Vibrio cholerae*, *Mycobacterium bovis*, *Shigella sonnei*, *Listeria monocytogenes*, and *Bacillus anthracis*; attenuated strains of these species have been constructed or isolated (Medina and Guzmán 2001). However, there is some risk of reversion to a pathogenic status. This risk could be avoided by using nonpathogenic bacteria, such as LAB. Besides their Generally Recognized as Safe category, some LAB have the capacity to colonize the gastrointestinal tract or the genital mucosa of animals and humans, making them excellent candidates for oral vaccines. Oral immunization has, among other advantages, the capacity to induce immunity through mucosal surfaces. These surfaces constitute one of the principal entrance points and the first line of defense of hosts against attack by pathogenic organisms (Mercenier 1999).

Some tests of live vaccines have been conducted with *L. lactis*, *Streptococcus gordonii*, and *Lactobacillus* sp. (Medaglini et al. 1997; Chatel et al. 2001; Reveneau et al. 2002). In these trials, LAB functioned as vectors for the production and presentation of model antigens on mucosal surfaces. These LAB were able to stimulate a specific immune response in mice exposed via the intranasal and oral routes.

Other experiments were designed to use LAB, not only as antigen presenters, but also as mucosal adjuvants. In these cases, recombinant strains of *L. lactis* produced both the model antigens and interleukins 2 and 6 (IL2 and IL6); mucosal administration with these recombinant lactococci was shown to increase 10–15 times the antigen-specific immune response (Steidler et al. 1998). Various studies are under way, using *L. lactis* as a model microorganism, so that these “new potential uses” of LAB can become a reality. More details concerning the use of LAB as live vaccines are presented in this chapter.

#### 9.4. *Lactococcus lactis*: The Model LAB

*L. lactis* is a facultative and mesophilic heterofermentative bacterium (ideal growth temperature around 30°C) that is widely used in the dairy industry. Currently, *L. lactis* is the best characterized member of the LAB group and is considered the model organism of this group, not only because of its economic importance, but also because of the following features: (1) it has a completely sequenced genome (Bolotin et al. 1999); (2) it is genetically easy to manipulate; and (3) many genetic tools have already been developed for this species (de Vos 1999).

##### 9.4.1. The Genetic Organization of *L. lactis*

Genetic studies on *L. lactis* have been mainly conducted on two strains: *L. lactis* MG1363 (formerly *L. lactis* subsp. *cremoris*) and *L. lactis* IL1403 (formerly *L. lactis* subsp. *lactis*).

*The genome.* Analysis of the organization and comparison of the genome sequences of these two subspecies of *L. lactis* demonstrated that they have approximately 80% identity and that their genomes have large inversions (Le Bourgeois et al. 1995). These studies also showed that the genome of *L. lactis* MG1363 has mobilization genes different from *L. lactis* IL1403, which corroborates findings that the former species contains plasmids that permit growth in a wide range of environments.

Recently sequenced (Bolotin et al. 2001), the genome of *L. lactis* IL1403 has 2.36 mega bases and 35.4% G+C content. A total of 2310 genes was identified, of which 86% code for proteins; 1.4% code for RNA; and 12.6% are noncoding sequences. Two hundred and ninety-three genes from six prophages and 43 insertion sequences were also identified. The genome of *L. lactis* also has four operons containing competence genes, which means that this bacterium, like *Bacillus subtilis* and *Streptococcus pyogenes*, would be able to go through natural DNA transformation. New information generated from sequencing the genome of *L. lactis* is already aiding in the development of new strains of bacteria in the construction of expression systems for the production of biotechnologically useful heterologous proteins and could, in the near future, improve commercial processes that involve bacteria, such as cheese aging.

*The plasmids.* As for other members of the LAB group, *L. lactis* can occupy a wide range of niches. The capacity to grow in environments such as milk is mainly due to genes found in plasmids that code for various proteins, including enzymes, which are essential for the metabolism of sugars and proteins, as well as bacteriocins and DNA-repair proteins (Duwat et al. 2000). The finding of these genes in extra-chromosomal DNA suggests that these abilities arose through horizontal transfer from phylogenetically related species, such as *S. thermophilus* (Guedon et al. 1995). This hypothesis is partly confirmed by the finding of the *cluA* gene in *L. lactis* MG1363. The protein coded by this gene possesses 1243 amino acids and has homology with aggregation proteins previously described in other Gram (+) bacteria such as *Enterococcus* sp. and *Streptococcus* sp. (Godon et al. 1994). The aggregation proteins are responsible for the initiation of bacterial conjugation, during which there is contact between the donor and receptor cells.

*Control of gene expression.* Gene transcription begins when the sigma ( $\sigma$ ) subunit of RNA polymerase recognizes a specific region of the DNA. This region, called the “promoter,” is located



upstream of a coding sequence or operon and is characterized by two sequences of hexanucleotides located around positions  $-35$  (TTGACA) and  $-10$  (TATAAT) relative to the site of the initiation of transcription. After the hexanucleotides are recognized, transcription begins.

Various promoters have been described for *L. lactis*, by systematic research (Kuipers et al. 1997) and by analyzing already identified genes. They have similar  $-35$  (TTGACA) and  $-10$  (TATAAT) sequences to those found in *Escherichia coli* and *B. subtilis* and a TG (thymine-guanine) motive is located one base pair before the sequence  $-10$ . The principal  $\sigma$  factor of *L. lactis*,  $\sigma^{39}$ , is coded by the *rpoD* gene (Araya-Kojima et al. 1995; Bolotin et al. 2001) and has homology with factors  $\sigma^{70}$  and  $\sigma^A$  from *E. coli* and *B. subtilis*, respectively. When the genome of *L. lactis* IL1403 was sequenced, a second sigma factor was identified (Bolotin et al. 2001). Transcription terminates at the 3' end of the genes and operons, where a palindromic sequence of nucleotides rich in guanine and cytosine and thymine (T), called "transcriptional terminators," signals the end of the process. Most of the genes and operons of *L. lactis* have such sequences.

In *L. lactis* the signals for the initiation of translation are similar to those already described for *E. coli* and *B. subtilis*. The ribosome-binding site is complementary to the 3' sequence of rRNA 16S (3' CUUCCUCC 5') of *L. lactis* (Chiaruttini and Millet 1993). Although most of the initiation codons are AUG, there are other codons, such as GUG (van de Guchte et al. 1992).

## 9.5. Genetic Tools for the Production of Heterologous Proteins in *L. lactis*

The utilization of *L. lactis* as a "bioreactor" for the production of biotechnologically relevant proteins is a result of an accumulation, over the last 20 years, of knowledge concerning microbiology, biochemistry, immunology, and mainly, genetics. Numerous genetic tools have been developed and some are currently being used in *L. lactis* and in other species of LAB.

Studies based on the identification and isolation of wild-type plasmids from *L. lactis* and other LAB have made it possible to develop various cloning vectors. Using molecular biology techniques, these plasmids have been manipulated so that they have become important tools for cloning and studying genes of interest, both those of prokaryotes and eukaryotes. They basically consist of (1) origin of replication (*ori*), (2) selection marker (gene) for antibiotic resistance, and (3) multiple-cloning site.

Among the vectors that are available, two have been intensively utilized: pAM $\beta$ 1 and pWV01 (Janni re et al. 1993). Vector pAM $\beta$ 1 is a plasmid derived from *Enterococcus faecalis*, with a "theta" ( $\theta$ ) form of replication. Currently, low- and high-copy number versions are available, such as the vectors pIL252 and pIL253, respectively (Simon and Chopin 1988). On the other hand, the plasmid pWV01, from *L. lactis* subsp. *cremoris*, undergoes rolling-circle type replication. Different from pAM $\beta$ 1, pWV01 shows a wide spectrum of hosts that range from LAB to Gram (−) microorganisms, such as *E. coli*. They also have low- and high-copy number versions, such as the vectors pGK1 and pGK12, respectively (Kok et al. 1984).

## 9.6. Expression and Targeting Systems of Heterologous Proteins

The expression of heterologous proteins in *L. lactis* has been favored both by advances in genetic knowledge and by new developments in molecular biology techniques. Using this duet of tools to obtain increased levels of these proteins and control their production, various vectors containing constitutive or inductive promoters were developed and currently constitute the basis of all expression systems in *L. lactis* and other LAB.

### 9.6.1. Expression Systems Based on the *Lac* Operon

One of the first expression systems for use in *L. lactis* was based on the promoter ( $P_{lac}$ ) and the regulator gene (*lacR*) of the *lac* operon. This operon causes the promoter  $P_{lac}$  to be induced (5–10 times)

in the presence of lactose; transcription of the regulating gene (*lacR*) is repressed at the same rate (van Rooijen et al. 1992). However, a higher level of induction was needed for this system to be useful for protein production. Consequently, a new system, composed of three vectors, which combined the elements of the *lac* operon with elements of bacteriophage T7 (phage T7) of *E. coli*, was developed (Wells et al. 1993b). In this new system, the open reading frame (ORF) that codes for RNA polymerase of phage T7 (T7 RNA pol) was placed under the control of the  $P_{lac}$  promoter on the vector pILPol and the ORF of interest was placed in a second vector under the control of the T7 promoter. This way, when lactose is added to the culture medium,  $P_{lac}$  induces the expression of the T7 RNA pol that activates the expression of the ORF of interest controlled by the T7 promoter. However, in order for the cell to be able to metabolize lactose in the medium, a third vector containing the *lac* operon is necessary. This system was first tested with fragment C of tetanus toxin (TTFC), a highly immunogenic model antigen, and was found to be capable of producing 22% of TTFC total cellular proteins. Mice immunized with *L. lactis* strains producing TTFC with this system were protected against lethal doses of tetanus toxin. Although it permitted control of gene expression and high levels of production, this expression system proved unviable because it consisted of three different vectors and three markers for resistance to antibiotics; the use of antibiotic markers also made it unviable for application in the food and pharmaceutical industries.

### 9.6.2. Expression Systems Based on Phage Promoters

Studies on the regulation of gene expression of phages infecting *L. lactis* MG1363 were the basis for the development of simpler expression systems, such as the “repressor-operator system” of the  $\lambda$ 1 phage of *L. lactis* (Nauta et al. 1996). In this system, and in the same vector, an ORF is placed under the control of the phage promoter  $P_{orf5}$  that is repressed by the phage protein Rro. When the mutagenic agent mitomycin C is added to the medium, the repressor

protein, Rro, is proteolytically cleaved, with consequent release of the promoter  $P_{orf5}$ . Free from repression, the promoter induces the expression of the ORF that it controls. This system was tested using the reporter gene *lacZ* of *E. coli* and subsequently using the *acmA* gene (autolysin) of *L. lactis* MG1363. However, the use of mitomycin C as an inducer impedes the use of this system for the production of proteins in fermentors or in food products.

In another system, the genetic elements of phage  $\phi$ 31 were used to develop an expression system that combines the  $P_{15A10}$  promoter and the origin of replication (*ori31*) (O’Sullivan et al. 1996). Here, as in the other systems, the ORF to be activated is cloned under the control of  $P_{15A10}$ , with the *ori31* on the same vector. After initiation of infection by the  $\phi$ 31, *ori31* is the target of the phage replication machinery and the number of copies of the vector in the cell is increased. Due to this increase, with the help of the  $P_{15A10}$  promoter, the level of expression is also increased. After cell lysis, caused by phage replication, the protein of interest is released into the medium. The greatest disadvantage of this system is the need for cell infection to obtain induction; this leads to the destruction of the cell culture, making the system unviable for industrial use.

### 9.6.3. The Nisin-Controlled Expression System

One of the most promising and powerful expression systems that have been developed for use in LAB is based on genes involved in the biosynthesis and regulation of the antimicrobial peptide, nisin. Nisin is a small peptide produced and secreted by various strains of *L. lactis*, which is widely used as a natural preservative, due to its antimicrobial properties. Eleven genes (*nisABTCIPRKFE*G) are responsible for the production, modification, and secretion of this peptide. The *nisA* gene encodes the 57-amino acid peptide precursor, nisin A; *nisBC* genes are involved with posttranslational modifications; *nisT* is needed for transport via the cytoplasmic membrane, while *nisP* is involved in the breakdown of the nisin precursor. The *nisI* gene, together with the *nisFEG* genes, codes for a lipoprotein that confers

to the bacteria immunity against the nisin that it produces. Finally, *nisR* and *nisK* code for a two-component system (NisRK) that controls the expression of the 11 genes via signal transduction. NisK functions as a membrane sensor that detects the presence of extracellular nisin. The signal is subsequently transferred to NisR through a phosphorylation process that activates it. NisR is then able to activate gene transcription controlled by the promoters  $P_{nisA}$  and  $P_{nisF}$  (Kuipers et al. 1993).

Based on this information, expression systems induced by nisin have been developed. Various expression vectors containing the  $P_{nisA}$  promoter, followed by multiple-cloning sites for the insertion of ORFs to be expressed, are now available. These systems can be used in bacterial strains containing only the *nisR* and *nisK* genes in the chromosome, and the nisin concentration necessary for the induction of expression is minimal (0.01–10 ng/ml). Alternatively, *L. lactis* NZ9700 can be used as a nisin producer to express constitutively a specific protein (de Ruyter et al. 1996; Kuipers et al. 1998).

The nisin-controlled expression (NICE) system has been tested in other LAB, such as *Leuconostoc lactis* and *Lactobacillus helveticus*, demonstrating its versatility. As these bacteria do not have *nisRK* genes in their genomes, a two-vector system was developed (Kleerebezem et al. 1997) in which the  $P_{nisA}$  promoter is fused to the specific ORF in one vector, and the *nisRK* genes are in a second vector. Later, this system was also used in other bacteria, such as *Streptococcus* sp., *Bacillus* sp., *Enterococcus* sp. (Eichenbaum et al. 1998), and *Lact. plantarum* (Pavan et al. 2000). Various heterologous proteins have already been expressed using this system; among all the systems that have been developed, this is the easiest to use and the one that gives the best yields.

#### 9.6.4. The P170 Expression System

Several lactococcal promoters regulated by environmental conditions have also been isolated. Among them, P170 is a natural and strong promoter of an uncharacterized gene termed *orfX*, only active at low pH (pH < 6) and when cells enter the stationary

growth phase of glucose-grown cultures (Madsen et al. 1999; Bredmose et al. 2001). P170 induction depends on RcfB, a positive regulator of the CRP-FNR family of transcription regulators that binds to ACiD-boxes (Körner et al. 2003; Madsen et al. 2005). For protein production, P170 offers the major advantage (compared with the NICE system) of self-inducibility via lactic acid accumulation during growth. Using P170, the Staphylococcal nuclease (Nuc; Shortle 1983) yield of 300 mg/l has been achieved (Israelsen et al. 1995; Madsen et al. 1999).

### 9.7. Cellular Targeting Systems of Heterologous Proteins

A series of studies to develop new strains and efficient expression systems has been conducted to use LAB as “cell factories” for the production of proteins (Djordjevic and Klaenhammer 1998). However, in order for some of these proteins (enzymes and antigens) produced by bacteria to attain the desired biological activity levels, it is necessary that they correctly target specific cellular locations: (1) cytoplasm, (2) membrane, or (3) extracellular environment.

In bacteria, protein targeting is accomplished via protein sequences or motifs. Among these, the signal peptide (SP) is a hydrophobic, poorly conserved, negatively charged motif, located at the amino-terminal (N-terminal) end of proteins naturally secreted by the cell. It is recognized and cleaved by the secretion machinery, which allows the protein to be transferred through the membrane and released into the extracellular medium. Another key sequence is cell wall anchoring signal, which is composed of 30 amino acids and is located at the carboxy-terminal (C-terminal) end of the protein. The cell wall anchor (CWA) includes a conserved motif (LPXTG), which is recognized by the anchoring machinery. The protein, translocated to outside the cell, is covalently bound by the LPXTG motif to a glycan peptide. Although these motifs, along with secretion and anchoring mechanisms, were first characterized in *B. subtilis* (Simonen and Palva 1993) and *Staphylococcus aureus* (Ton-That et al. 1999), homologous systems are present in other

Gram (+) bacteria, such as LAB (Fischetti et al. 1990).

Consequently, studies have been conducted to better understand and characterize the factors that participate in and influence the secretion of homologous and heterologous proteins in LAB. It is now known that few proteins are secreted in *L. lactis*; among these, Usp45 was found. This protein, whose function is unknown, is the only one secreted in quantities large enough to be detected in Coomassie stained protein gels (van Asseldonk et al. 1990). It is also known that *L. lactis* is able to recognize secretion (SP) and anchoring (CWA) motifs of bacteria such as *S. aureus* and *S. pyogenes* (Le Loir et al. 1994; Piard et al. 1997). Based on this knowledge and on previously developed expression systems, some expression and targeting systems of heterologous proteins have been constructed.

One of the first expression and targeting systems of heterologous proteins constructed to be used in *L. lactis* was based on the T7 RNA pol system. In this system, the T7 promoter is fused to the DNA fragment that codes for the signal peptide of the Usp45 protein (SP<sub>Usp45</sub>), followed by the TTFC ORF. After induction of the system, the transcriptional fusion (*sp<sub>Usp45</sub>::TTFC*) was efficiently translated and the TTFC was secreted to the extracellular environment in significant quantities (2.9 mg/l; Wells et al. 1993a).

A second, simpler and more efficient expression and targeting system was developed (Dieye et al. 2001) using elements such as the constitutive promoter P<sub>59</sub> (*Streptococcus cremoris*; van der Vossen et al. 1987), SP<sub>Usp45</sub>, and the anchoring motif CWA of *S. pyogenes* M6 protein. This system made it possible for the model protein Nuc (*S. aureus* nuclease; Shortle 1983) to be directed to the cytoplasm, membrane, and extracellular medium. For cytoplasmic production, the 5' end of the *nuc* ORF was simply fused to the P<sub>59</sub> promoter. In order to anchor Nuc to the membrane, the 5' and 3' ends of *nuc* were respectively fused to *sp<sub>Usp45</sub>* and *cwa<sub>M6</sub>*. Finally, in order for Nuc to be exported to the extracellular medium, only a transcriptional fusion between *sp<sub>Usp45</sub>* and *nuc* was necessary. Although this system made it possible to target Nuc to three different cel-

lular compartments and was used successfully in *L. lactis* subsp. *cremoris* and *Lact. plantarum*, the protein production levels were low (2.5–3 mg/l), probably due to the low copies of the vector and to the extracellular protease, HtrA (Poquet et al. 2000).

Therefore, a third system was constructed whose objective was not only to target heterologous proteins to outside the cell, but also to control and increase gene expression (Le Loir et al. 2001). In a previous study (Le Loir et al. 1998), it was found that the efficiency of Nuc protein secretion was low (3 mg/l), probably due to the positive charges (+3) of its own signal peptide. A synthetic propeptide, LEISSTCDA, which has a negative charge (−2), was developed. When fused to the coding sequence of the N-terminal end of Nuc, this propeptide was able to increase the efficiency of secretion of Nuc five times (15 mg/l). In a second stage, in order to increase the levels of expression, the coding sequence of the signal peptide of Usp45 (*sp<sub>Usp45</sub>*) was cloned together with the *nuc* ORF in a high-copy number vector, under the control of the P<sub>nisa</sub> promoter. Next, in order for Nuc to be efficiently recognized by the cell secretion machinery, the coding sequence of the synthetic propeptide LEISSTCDA was cloned between *sp<sub>Usp45</sub>* and the 5' end of *nuc* ORF (P<sub>nisa</sub>::*sp<sub>Usp45</sub>::LEISSTCDA::nuc*); this resulted in high levels of Nuc in the extracellular medium (25 mg/l). Currently, this system has been used successfully to express and target various biotechnologically important heterologous proteins and it is among the best genetic tools available (Nouaille et al. 2003; Le Loir et al. 2005).

Another expression and targeting system that figures among the ones previously mentioned is the xylose-inducible expression system, which is based on the use of a xylose-inducible lactococcal promoter, P<sub>xyIT</sub> (Jamet 2001). The capacity of this system to cytoplasmic and secreted proteins was initially tested using the coding sequence of the Staphylococcal nuclease (*nuc*) fused or not to the lactococcal Usp45 signal peptide coding sequence. Xylose-inducible *nuc* expression was found to be tightly controlled and resulted in high-level, long-term protein production and targeting either to cyto-

plasm or the extracellular medium. Furthermore, this expression system is versatile and can be easily switched on or off by adding either xylose or glucose, respectively (Miyoshi et al. 2004).

Recently, a new expression system called  $P_{Zn}$  *zitR* has been developed in *L. lactis*. With excess zinc in the medium, ZitR binds to  $P_{Zn}$  and represses expression by competing with RNA polymerase binding. Alternatively, under extreme zinc starvation, ZitR becomes inactive and allows RNA polymerase binding to initiate  $P_{Zn}$  transcription. This lactococcal expression/secretion system uses a consensus signal peptide, SP<sub>Exp4</sub> that allowed efficient production of Nuc and  $\beta$ -galactosidase. This production process is of particular interest because it is inexpensive and compatible with large-scale production (Llull and Poquet 2004; Morello et al. 2008).

## 9.8. Genetically Modified Strains

One of the biggest problems found in the production of specific proteins in wild-type strains is low yield, due to intrinsic cellular factors, such as (1) instability of the messenger RNA, (2) toxicity of the recombinant protein, and mainly (3) protein instability. Protein instability is frequently attributed to intra- and extracellular proteolytic systems, found both in Gram (–) and Gram (+) bacteria. A variety of these systems have already been described in *E. coli* and *B. subtilis*; they involve diverse physiological functions, such as protein degradation, assimilation of nutrients such as peptides and amino acids, and degradation of proteins that are malformed and/or foreign to the cell (heterologous; Gottesman 1996). Unlike these bacteria, *L. lactis* has only one extracellular protease, HtrA (Poquet et al. 2000), which is responsible not only for the propeptide processing and maturation of wild-type proteins, but also for allowing cells to grow at high temperatures, such as 37°C. Inactivation of the *htrA* gene in *L. lactis* strain IL1403 demonstrated that this membrane protease is also the main enzyme responsible for degrading heterologous proteins. The results obtained with the production of specific proteins in this strain demonstrate that this mutant is capable of completely stabilizing these proteins, that is, no degradation due

to proteases was observed. We conclude that this *L. lactis* strain constitutes an important tool for the production of biotechnologically useful proteins.

Another important element in the production of heterologous proteins is the selection markers (genes for resistance to antibiotics). Present in cloning and expression vectors, these types of markers are widely used in laboratories to select and maintain cells that carry recombinant plasmids. However, their use in the food and pharmaceutical industries is not considered acceptable due to the presence of antibiotics in the final product and because of contamination of the environment. Currently, this problem is being overcome by constructing auxotrophic bacterial strains, the deficiencies of which are corrected by including the wild-type gene in a cloning or expression vector (Henriksen et al. 1999). The first selection system that did not include antibiotics was based on complementation of a chromosomal gene *lacF* of the *lac* operon. Expression of the *lacF* gene in a cloning vector resulted in complementation of the *lac*<sup>–</sup> phenotype, allowing selection of clones carrying this vector in culture medium supplemented with lactose (Dickely et al. 1995). Since then, other auxotrophic strains of *L. lactis* have been developed, leading to the establishment of “food-grade” systems that could be used to produce proteins directly in food or in large-scale fermentations, without selection markers.

Two systems based on threonine- and pyrimidine-auxotroph derivative *L. lactis* strains allow the cloning and efficient expression of heterologous and homologous proteins in various industrial strains (Sorensen et al. 2000; Glenting et al. 2002). These two systems are stable, and do not impair growth and important properties, such as milk acidification. Because of the absence of selection markers and foreign DNA, strains using these systems maintain their food-grade status.

A large number of expression vectors are now available in *L. lactis* with different strengths and regulation systems, some of them exerting strong activity and tight regulation. They constitute powerful tools to control heterologous protein production in terms of quantities, timed expression, and conditions.



## 9.9. LAB as Live Vaccines

Mucosal epithelia constitute barriers between the internal and the external environment and consequently are the first line of defense of animals against most of the pathogens that use this way of entry (Salminen et al. 1998). Therefore, strategies designed to fight against pathogens at mucosal surfaces are not only desirable but, in some instances, can be the only way to prevent infection. Recent advances in biotechnology and in the understanding of the immune system have now made it possible to develop new mucosal vaccines based on live bacterial vectors able to stimulate mucosal immunity.

Among the live bacterial vectors, two main types can be distinguished: those based on attenuated pathogens and those based on nonpathogenic bacteria (Brahmbhatt et al. 1992; Stevenson and Roberts 2003). Attenuated pathogenic bacteria are particularly well adapted to interact with mucosal surfaces as most of them use this portal to initiate infection. Unfortunately, these organisms could recover their pathogenic potential and are not totally safe for use in humans, especially in children and immunosuppressed patients.

Therefore, LAB would be an attractive alternative to attenuated pathogenic bacteria (Stahl et al. 1997; Lee 2003). In addition to their GRAS status, some LAB are able to stimulate the immune system of the host as adjuvants due to their probiotic properties and their immunomodulation capacity (Seegers 2002). Furthermore, LAB are poorly immunogenic, in contrast to pathogenic microorganisms that are themselves highly immunogenic, a feature that enables the repetitive use of the carrier in multi-schedule immunizations with the same or other antigens (Robinson et al. 1997; Chang et al. 2003).

The combination of these properties makes LAB very advantageous live vaccines, and various molecular tools have been developed to efficiently express antigens and therapeutic molecules at different cellular localizations in LAB (Wells et al. 1993b; Norton et al. 1995; Le Loir et al. 2001). Strikingly, mucosal administration with genetically engineered LAB has been shown to elicit both systemic and

mucosal immunity (Robinson et al. 1997; Chang et al. 2003).

In particular, among the LAB, *L. lactis* is considered a potential candidate for the development of new safe mucosal vaccines because many genetic tools have been developed for it and because its complete genome is sequenced. However, the major advantage of the use of *L. lactis* as live vector for mucosal delivery of therapeutic proteins resides in its extraordinary safety profile since this bacterium is cataloged as a noninvasive and nonpathogenic organism (Salminen et al. 1998).

The first attempt to study the potential of *L. lactis* as a mucosal vaccine was performed with killed recombinant lactococci producing a cell wall-attached form of a protective antigen (PAC) from *Streptococcus mutans*. Mice immunized orally with this recombinant strain developed a PAC-specific serum IgG and mucosal IgA antibodies (Iwaki et al. 1990). These results showed, for the first time, that *L. lactis* could be used as a delivery vector to present an antigen to the immune system.

The majority of studies using *L. lactis* as a live vector uses TTFC. Norton et al. (1995) observed a significant increase in the level of IgA after oral immunization in mice with recombinant strains producing TTFC. Other studies showed that mice immunized with recombinant strains of *L. lactis* producing intracellular TTFC develop significantly higher levels of IgG and TTFC-specific fecal IgA. These mice become more resistant to a lethal challenge with the tetanus toxin than do nonimmunized mice (Wells et al. 1993b; Robinson et al. 1997; Grangette et al. 2001).

Nevertheless, the most promising results were observed in *L. lactis* expressing cell wall-anchored E7, a major candidate antigen for vaccines against HPV-related cervical cancer, and a secreted form of interleukin-12 (IL-12). Therapeutic immunization with these *L. lactis* strains, after TC-1 injection, induced regression of palpable tumors in treated mice (Bermúdez-Humarán et al. 2005). These pre-clinical results suggest the feasibility of mucosal vaccination and/or immunotherapy against HPV-related cervical cancer using genetically engineered lactococci.

**Table 9.1.** Protection studies with *L. lactis* based vaccines (Wells and Mercenier 2008; modified).

Vaccine target	Antigen (model)	Model (route)	Immune <sup>a</sup> responses	Protection model (outcome)
<i>Streptococcus pneumoniae</i>	PspA	Mouse; intranasal	Serum antibody and BALF antibody	Infectious lethal challenge intraperitoneally (increased survival)
<i>Streptococcus pyogenes</i>	C- repeat region of M protein serotype 6 (cell wall associated)	Mouse; intranasal and subcutaneous	Salivary IgA and serum antibody	Pharyngeal infection (intranasal route; protective)
HIV-1	V2-V4 loop of gp 120 (cell wall associated)	Mouse; intragastric with cholera toxin adjuvant	Serum antibody, fecal antibody, ICCS, tetramer assay, and ELISPOT	Intraperitoneal challenge with HIV-1 Env-expressing vaccinia virus (viral load reduced)
<i>Erysipelothrix rhusiopathiae</i>	SpaA (cell wall associated)	Mouse; intranasal	Serum antibody and fecal IgA	Challenge with <i>E. rhusiopathiae</i> (protection from death)
Rotavirus	VP7 (cytoplasmic, cell wall associated and secreted)	Mouse; intragastric	Serum antibody	Virus neutralization assay (neutralizing antibody demonstrated for VP7; cellwall-associated vaccine)
Group B <i>Streptococcus</i>	Pilus (island 1) (cell-wall associated)	Mouse; subcutaneous, intraperitoneal and intranasal	Serum antibody and antibodies in nasal and vaginal washes	Survival of offspring from vaccinated mothers after infectious challenge
<i>Brucella abortus</i>	L7 or L12 (cytoplasmic)	Mouse; intragastric	Fecal IgA	Partial protection against intraperitoneal inoculation of virulent <i>B. abortus</i>

<sup>a</sup>Responses detected using any of the indicated vaccination routes.

BALF = bronchoalveolar lavage fluid, CTL = cytotoxic T lymphocyte, ELISPOT = enzyme-linked immunospot, ICCS = intracellular cytokine staining.

Although the studies of LAB as delivery vehicles have focused mainly on the development of mucosal vaccines, these microorganisms have also been used as a delivery system for a range of molecules including allergens, enzymes, and cytokines (Wells and Mercenier 2008). Clinical trials have revealed that interleukin-10 (IL-10) can reduce diarrheic disorders associated with inflammatory bowel disease. In mice, gastric administration of *L. lactis* secreting IL-10 reduced diarrheic symptoms by about one-half and also prevented their development (Steidler et al. 2000). This genetically engineered strain has been approved by Dutch authorities to be used as an experimental therapy for humans (Steidler et al. 2003). This unique trial opens the possibility of the use and acceptance in humans of recombinant lactococci for vaccination and/or therapy.

In the last 10 years, the capacity of *L. lactis* to produce antigens has been clearly demonstrated

(Table 9.1) and in some cases, mucosal administration with these recombinant lactococci was shown to induce a successful mucosal and systemic immune response (Xin et al. 2003; Robinson et al. 2004).

The capacity of the genus *Lactobacillus* to produce antigens has also been demonstrated (Table 9.2). The first studies proposing the use of genetically modified lactobacilli to produce heterologous proteins with the aim of developing a new generation of mucosal vaccines were carried out by Pouwels et al. (1996) and Rush et al. (1994). By the end of the 1990s and early 2000s, several laboratories demonstrated interest in the use of recombinant lactobacilli (particularly *Lact. casei* and *Lact. plantarum*) as vehicles to deliver clinically relevant proteins at mucosal surfaces and stimulate both strong and local immune responses (Kruisselbrink et al. 2001; Chancey et al. 2006; Poo et al. 2006; Wu and Chung 2007; Campos et al. 2008; Yigang and Yijing

**Table 9.2.** Protection studies with lactobacilli based vaccines (Wells and Mercenier 2008; modified).

Vaccine target	Vehicle	Antigen (model)	Model (route)	Immune <sup>a</sup> Responses	Protection model (outcome)
<i>Helicobacter pylori</i>	<i>Lact. plantarum</i> and <i>Lact. plantarum</i> alr	Urease B (cytoplasmic)	Mouse; intragastric	Serum antibody	Colonization level (partial protection)
Tetanus	<i>Lact. plantarum</i> and <i>Lact. plantarum</i> alr	TTFC (cytoplasmic)	Mouse; intragastric, intranasal and intravaginal	Serum antibody, BALF, T cells and neutralizing antibody	Survival after tetanus toxin challenge (protection)
<i>Strep. pneumoniae</i>	<i>Lact. plantarum</i> and <i>Lact. helveticus</i>	PsaA	Mouse; intranasal	Antibody in serum, BALF and nasal wash	Nasal colonization (reduction in pneumococcal load)
Enterotoxigenic <i>E. coli</i>	<i>Lact. acidophilus</i>	K99 fimbriae	Pig; intestinal brush border <i>ex vivo</i>	Not applicable	Inhibition of K99 <i>E. coli</i> adhesion in porcine intestinal brush border
SARS—associated coronavirus	<i>Lact. casei</i>	Spike antigen segments	Mouse; intragastric and intranasal	Serum antibody and mucosal IgA	Viral neutralizing antibody elicited
HPV16—induced tumors	<i>Lact. casei</i>	E7	Mouse; intragastric	Serum antibody, mucosal IgA and ELISPOT	Protection demonstrated against injection of E7—expressing tumor cell line

<sup>a</sup>Responses detected using any of the indicated vaccination routes.

alr = alanine racemase mutant, BALF = bronchoalveolar lavage fluid, ELISPOT = enzyme-linked immunospot, HPV = human papillomavirus, SARS = severe acute respiratory syndrome, TTFC = tetanus toxin fragment C.

2008). Then, several peer-reviewed publications have been published, confirming the advantages of the genus *Lactobacillus* for live mucosal vaccines (Hanniffy et al. 2004; Mohamadzadeh et al. 2008; Wells and Mercenier 2008).

## 9.10. Conclusions

The utilization of LAB for the production of biotechnologically important heterologous proteins, as well as for oral vaccines, is a visionary view that each day becomes a reality. As more information concerning this bacterial group is generated, new possibilities for their use are being contemplated. We consider that a complete toolbox is now available for heterologous protein production and targeting in *L. lactis*, and will be extended to other LAB. These tools could lead to the construction of new food-grade live vaccines based on LAB. Such uses for vaccination purposes are promising for future therapeutic use of these bacteria. The most difficult

tasks with this type of vaccination are to produce each antigen or cytokine in the precise cell location (cytoplasmic, anchored, or secreted) and to regulate expression levels to induce the highest efficiency in immune response. For the moment, *L. lactis* is still the model LAB, and it is the easiest LAB to manipulate. Lactobacilli are being studied more and more because of their adjuvant properties, but in spite of these efforts, they are still more difficult to handle than lactococci. Two strategies should be followed in the future: (1) laboratory production systems based on antibiotic resistance genes will be replaced by food-grade systems much better accepted by potential consumers, and (2) cocktails will be used of recombinant lactococci producing antigens and cytokines, and lactobacilli as probiotic adjuvants.

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## Chapter 10

# Advances and Trends in Starter Cultures for Dairy Fermentations

Domenico Carminati, Giorgio Giraffa, Andrea Quiberoni, Ana Binetti, Viviana Suárez, and Jorge Reinheimer

*Dairy starter cultures are actively growing cultures of lactic acid bacteria (LAB) to drive the fermentation process. At first, only artisanal starters were used. Though they are still used in certain cases, their microbiological instability promoted an evolution to more defined mixtures of LAB in order to obtain a more stable acidifying activity and quality of the products. However, artisanal starters are valuable sources of new strains for commercial uses. Today, defined multi- or mono-strain cultures are extensively used around the world to produce cheeses, fermented milks, and cream butter. They are commercialized as frozen and freeze-dried cell concentrates, and Direct Vat Inoculation starters, to be used directly in the vats. Since phage sensitivity of lactic cultures constitutes a critical item when the starters are selected for industrial use, many strategies have been developed, among which the obtaining of phage-insensitive mutants or the conjugal transfer of resistance plasmids has been applied. Starter adjuncts, used for purposes other than acid formation, are receiving attention as ripening or protective cultures to improve the organoleptic characteristics of cheeses or perform a specific antimicrobial activity, respectively. Some probiotic LAB, such as *Lactobacillus casei/paracasei* and *Lactobacillus plantarum*, have demonstrated starter activity, so they can be recognized as “probiotic starters.” Fundamental and applied research is still needed to better improve starter cultures in the existing production technology and to obtain quan-*

*titative data that may yield precious information about the relationship between the cheese environment and bacterial functionality, thus contributing to optimal strain selection. The recent development of microarray technology and comparative genomics will allow a more complete genome analysis for a better selection of strains for specific uses.*

### 10.1. Introduction

The basic role of dairy starter cultures composed of lactic acid bacteria (LAB) is to drive the fermentation process. Concomitantly, they contribute to the sensorial characteristics of the products and to their safety. Since the starter industry relies on the use of selected strains of given species with known metabolic properties, the introduction of starter cultures has undoubtedly improved the commercial and hygienic quality of the products and the overall process of standardization. On the other hand, the limited number of available strains with high technological performance and the constant risk of bacteriophage attacks justify the continuous need to search for new strains for product diversification.

### 10.2. General Aspects

Dairy starter cultures are actively growing cultures of LAB that are added to milk to target the fermentation process. They are used in the production of a variety of dairy products including cheese,

fermented milks, and cream butter. Since the original lactic acid microflora of the milk is either inefficient, uncontrollable, and unpredictable, or is destroyed altogether by the heat treatments to which milk is subjected, a starter culture can provide a more controlled and predictable fermentation. The LAB starters are primarily used because of their capability to produce lactic acid from lactose. Moreover, other important functions like inhibition of undesirable organisms, improvement of sensorial and textural properties, as well as contribution to health benefits are recognized. In yogurt and fermented milks production, the high heat treatment of the milk strongly reduces the indigenous milk microflora; the starter strains becoming predominant and responsible for the fermentation process. In cheese making, although the starter maintains the role of addressing the fermentation process, residual strains from milk and the environment grow together with the starter strains.

The pH decrease during the fermentation of dairy products affects a number of aspects of the manufacturing process, including the quality, texture, and composition of the products. Lactic acid imparts a distinctive and fresh acidic flavor of fermented milks. The fermentation process has to be controlled (e.g., by proper cooling) to avoid excessive acid concentration that can mask more delicate flavor like diacetyl. The lactic acid in cheese making is responsible for the milk coagulation and the texturizing. The pH decrease indirectly affects flavor by controlling the proteolytic activity of both coagulant and natural milk proteinases, and by influencing the biochemical reactions involved in the formation of other flavor compounds.

The lactic starter bacteria are involved in the production of aroma compounds, which enhance the organoleptic properties and determine the specific identity of the cultured dairy products. The development of flavor compounds can derive from fermentation of lactose and citrate, from degradation of milk proteins and fat, and from amino acids and free fatty acids metabolism. The contribution of flavor to the final products depends on a large number of factors (e.g., specific metabolic characteristics of starter strains, milk fermentation conditions, product

type and composition, and storage or ripening conditions).

The acidic condition and the reduced pH of the fermented dairy products, as well as the antimicrobial activity of undissociated lactic acid molecules, prevent the growth or survival of many spoilage and pathogenic bacteria. The capability of a few strains to produce secondary metabolites having an inhibitory activity (i.e., bacteriocins such as nisin and other inhibitory peptides, hydrogen peroxide, diacetyl) can enhance the preservative effects. In addition to the influence of pH decrease, which enhances the whey expulsion from the coagulated curd and indirectly affects the cheese texture, some strains of LAB are able to produce exopolysaccharides that can improve texture characteristics to certain cultured dairy products. Exhaustive reference on general and specific aspects of starter cultures can be found in Broome et al. (2003), Powell et al. (2003), Parente and Cogan (2004), and Vedomuthu (2006).

### 10.3. Types of Starter Cultures

Starter cultures may be classified on the basis of their function, their temperature of growth, or their composition. Starter cultures may be practically categorized as mesophilic or thermophilic according to the incubation and manufacture temperatures under which they are used. Mesophilic cultures grow and produce lactic acid at an optimum growth temperature of about 30°C and can reach a maximum fermentation temperature of 38–40°C. The most used mesophilic LAB species are *Lactococcus lactis* subsp. *lactis*, *L. lactis* subsp. *lactis* biovar *diacetylactis*, *Lactococcus lactis* subsp. *cremoris*, *Leuconostoc mesenteroides* subsp. *cremoris*, and *Leuconostoc lactis*. Mesophilic cultures can be further classified on the basis of citrate fermentation and composition, as citrate-negative starters (which contain acid producing *L. lactis* subsp. *lactis* and *cremoris*) or citrate-positive (Cit<sup>+</sup>) starters (containing *Leuc. mesenteroides* subsp. *cremoris*, *L. lactis* subsp. *lactis* biovar *diacetylactis*, or both, in addition to acid-producing strains). Thermophilic cultures have an optimum growth temperature of about

42°C. The most used thermophilic LAB species are *Streptococcus thermophilus*, *Lactobacillus delbrueckii*, and *Lactobacillus helveticus*. Mixtures of mesophilic and thermophilic microorganisms can also be used as in the production of some cheeses.

Probably, the most common classification of starter cultures is based on the complexity of the culture and the way it is reproduced (Powell et al. 2003, Parente and Cogan 2004).

### 10.3.1. Undefined Strain Cultures: Artisanal Starters

All LAB starter cultures available today are derived from artisanal starters of undefined composition (i.e., containing an undefined mixture of different strains and/or species). The production of such cultures, also defined as “natural starters,” is derived from the ancient back-slopping practice (the use of an old batch of a fermented product to inoculate a new one) and/or by application of selective pressures (heat treatment, incubation temperature, low pH). No special precautions are used to prevent contamination from raw milk or from the cheese-making environment, and control of media and culture conditions during starter reproduction is very limited. As a result, even in any given cheese plant, natural starters are continuously evolving as undefined mixtures composed of several strains and/or species of LAB.

The production of many traditional cheeses in Europe still relies on the use of natural mixed strain starters, which are an extremely valuable source of strains with desirable technological properties (phage resistance, antimicrobials, and aroma production), although many strains show limited acid production ability when cultivated as pure cultures (Parente and Cogan 2004). Fluctuations in composition result in variable performance and this may not be acceptable in modern cheese-making practice. Natural starters are considered to be highly tolerant to phage infection because they are reproduced in the presence of phages, which leads to the dominance of resistant or tolerant strains.

Two subtypes of artisanal (natural) starters are recognized, whey starters and milk starters, depend-

ing on the substrate and the technique used for their reproduction.

*Natural whey cultures (NWC).* NWC are prepared by incubating some of the whey drained from the cheese vat overnight under more or less selective conditions. In the manufacture of Parmigiano Reggiano and Grana Padano cheeses, whey is removed from the cheese vat at the end of cheese making at 50–54°C and it is incubated overnight at a controlled temperature (45°C), or in large containers in which the temperature decreases to 37–40°C, to a final pH as low as 3.3. The resulting whey culture is dominated by aciduric and/or thermophilic LAB species belonging to *Lact. helveticus*, *Lact. delbrueckii*, and *Strep. thermophilus*. Similar whey cultures are used in the production of stretched (“pasta-filata”) cheese varieties in Italy, hard cheese varieties in Argentina, and Comte cheese in France. Other types of NWC include deproteinized whey starters used for the manufacture of Pecorino cheese, and deproteinized whey starters with rennet that are used for the manufacture of Swiss-type cheeses (Emmental, Sbrinz, Gruyere) in small cheese factories in the Alps. Invariably, thermophilic lactobacilli (*Lact. helveticus*, *Lact. delbrueckii* subsp. *lactis*) dominate cultures produced under selective conditions (high temperature) while streptococci (*Strep. thermophilus*, but also lactococci and enterococci) often dominate cultures incubated at a relatively low temperature (35–40°C), which usually show higher microbial diversity (Reinheimer et al. 1995; Parente and Cogan 2004; Mucchetti and Neviani 2006).

*Natural milk cultures (NMC).* NMC are still used in small cheese-making plants in both Southern and Northern Italy for the production of traditional cheeses, and less used in Argentina. The selective pressure used for the development of the desired microflora includes thermization/pasteurization of raw milk (62–65°C for 10–15 min) followed by incubation at 37–45°C until the desired acidity is reached. The methodology used to prepare them and the microbiological composition of the raw milk used determine their microflora. This is usually dominated by *Strep. thermophilus* but other LAB



such as enterococci (*Enterococcus faecium* and *Enterococcus faecalis*, mainly), lactobacilli (*Lact. helveticus*, *Lact. delbrueckii* subsp. *bulgaricus/lactis*, and *Lactobacillus casei* subsp. *paracasei*), and *Streptococcus macedonicus*, may be present (Reinheimer et al. 1997; Marino et al. 2003; Parente and Cogan 2004, Mucchetti and Neviani 2006, De Angelis et al. 2008). Concerning the non-lactic acid microflora of natural milk cultures, this is commonly integrated by yeasts (*Debaryomyces*, *Saccharomyces*, *Trichosporon*, *Candida*, and *Rhodotorula*; Lodi 1980; Carrasco et al. 2006), *Micrococcus*, and coliform bacteria (Reinheimer et al. 1997).

Natural milk cultures are valuable sources of wild *Strep. thermophilus* strains. There is a permanent interest for obtaining new *Strep. thermophilus* isolates to be included in defined commercial starters as this species is used the most for cheese and fermented milks production.

### 10.3.2. Undefined Strain Cultures: Mixed-Strain Starters

For many cheeses, artisanal starters have been replaced by commercial mixed-strain starters (MSS), derived from the “best” natural starters and reproduced under controlled conditions by specialized companies and distributed to cheese plants, which use them to build up bulk starter or for direct vat inoculation (see below). While the composition of MSS is undefined, their reproduction under more controlled conditions reduces the intrinsic variability associated with the use of artisanal starters (Limsowtin et al. 1996). The traditional method for the reproduction of MSS, which requires several transfers in the cheese-making plant to build up the bulk starter by using small amounts of stock cultures, has been replaced by the use of concentrated cultures for the inoculation of the bulk starter tank or for direct inoculation of the cheese milk, thus minimizing the need for transfers within the factory and the risk of fluctuations in starter composition and activity. Either mesophilic or thermophilic MSS can be used in cheese making (Parente and Cogan 2004).

### 10.3.3. Defined (Multi)strain Cultures (DCS)

DSC allow greater control over the composition and properties of the culture. Examination of key properties of each strain (e.g., genetic or biochemical features, growth, and acid production characteristics) can lead to rational mixing of strains to formulate a culture with a desirable set of properties. DSC are composed of one or more strains selected, maintained, produced, and distributed by specialized companies. Because of their optimized, highly reproducible performance and high phage resistance, DSC have replaced traditional starters in the production of many cheese varieties, including some Protected Designation of Origin European varieties. Since the strain and/or species ratio in DSC is defined, their technological performance is extremely reproducible. This is a desirable property in modern cheese plants with large throughputs of milk and tight production schedules. However, as a consequence of the limited number of strains used, a phage infection may cause the disruption of lactic acid fermentations. On the other hand, with the subsequent loss of natural microbial diversity, the maintenance of the typical features is difficult. Strains diversity in cheeses has decreased with this method (Marino et al. 2003). These commercial starters are normally integrated by strains that were not isolated from the geographical area where cheeses are produced. However, there are many studies aimed at replacing natural starters by a defined (multi)strain culture that includes selected strains isolated from those specific geographical areas. These studies were performed for Italian (Carminati et al. 1985; Bossi et al. 1986; Gatti et al. 1993; Randazzo et al. 2006, 2007; de Candia et al. 2007; Pisano et al. 2007; De Angelis et al. 2008), Spanish (Menéndez et al. 2000), Portuguese (Macedo et al. 2004), and Argentinian (Candioti et al. 2002) cheeses.

Also for Kefir (acid-alcoholic fermented milk) the use of a commercial starter culture was assayed (García Fontán et al. 2006). Some parameters (e.g., microbial counts, residual lactose and ethanol content, and final pH) were similar to those obtained when a natural starter was used. However, other parameters, such as the yeast count after 48 h of

fermentation and titratable acidity, were slightly higher than those found in autochthonous products.

These aforementioned studies show that replacement of natural cultures is not always an adequate alternative for obtaining a final product with similar sensorial characteristics to traditional fermented foods. However, the use of multi-strain defined cultures is encouraged to reach a uniform, high-quality final fermented product.

*Direct vat inoculation (DVI) cultures.* DVI cultures are DSC grown in large volumes by the culture supplier, concentrated (typically by centrifugation) and then frozen or freeze-dried for storage and transportation to the cheese factory. The concentrated culture is added directly to the vat, without need for on-site starter propagation. DVI starters have become more accessible in recent times as the technology for concentration and preservation has been improved.

DVI can provide advantages, especially for factories that lack the specialized facilities and trained personnel necessary for reliable bulk starter preparation. Under ideal conditions of hygiene and milk composition stability, consistent cheese manufacture can be achieved because the starter inoculum can be pretested and standardized. Starter propagation and concentration by an external supplier reduces the probability of contamination of the starter by phages from the cheese factory environment. All types of cheese and fermented milk drinks can be made using DVI primary cultures. The extent of usage is limited by the operation scale and types of cheese made. In general, cheeses that require rapid production of lactic acid during the first 2–3 h (e.g., Cheddar) require a high inoculum level. In these cases, the use of DVI is very expensive. Typically, any plant producing more than 10,000 tons of cheese per year would find the use of DVI a major cost item. On the other hand, smaller plants would find DVI more convenient and more economical than bulk starter.

Secondary “adjunct” cultures, which are used to control or enhance the secondary fermentations in cheese, are ideally suited to be supplied in DVI form. These cultures will be discussed later.

## 10.4. New Sources of Primary Starters

The use of industrial starters has reduced the diversity of fermented dairy products. This phenomenon can be explained by the fact that the commercial availability of new interesting starter cultures is limited. Nowadays, there is an effort in searching for potential starter organisms from the pool of wild strains recoverable from raw milk, undefined cultures, or traditional cheeses. NWC for hard cheeses could represent a source of interesting isolates since they often contain (1) LAB strains with dairy-grade (e.g., antibiotic susceptibility) or pro-technological (e.g., broad phage resistance) traits (Rossetti et al. 2009) and (2) lactobacilli, in particular *Lact. helveticus*, which have strong proteinase and peptidase activities that influence cheese quality and are involved in the production of bioactive peptides from casein (Korhonen and Pihlanto 2006; Broadbent et al. 2008).

Many starter bacteria used in defined cultures are phage-related, implying that the number of different strains is generally limited. Efforts have been made to isolate “new” strains from raw milk, plants, and other natural sources (Wouters et al. 2002; Parente and Cogan 2004). Any potential new starter strain must produce acid rapidly, lack off-flavor development in milk, and be phage-resistant. *L. lactis* subsp. *lactis* strains but not *L. lactis* subsp. *cremoris* strains have been isolated from various vegetables, and many of them were good acid producers, coagulating milk in 18 h at 21°C. In contrast, very few strains of *L. lactis* isolated from artisanal dairy products were good acid producers (Parente and Cogan 2004). Some of them produce unusual flavors in milk. For example, the combination of a “wild” strain, which had low proteolytic activity and high amino acid decarboxylase activity, with a commercial strain, which had high proteolytic activity and low decarboxylase activity, resulted in the production of chocolate flavor in milk, due to the presence of several branched chain aldehydes and acids (Wouters et al. 2002). This latter example underlines the difficulty in designing appropriate starter formulations, which is an important task for the starter industry.

## 10.5. Specialized Cultures of LAB

Searching for strains with interesting properties to be used as new, specialized starter cultures may lead to an improved fermentation process and an enhanced quality of the end-product. In this regard, the use of specialized LAB cultures with expanded characteristics is promising in dairy technology. According to the specific function to be accomplished, specialized LAB cultures can belong to either the primary starter (e.g., the phage-resistant starters) or a secondary culture (the latter being also defined as “starter adjuncts”).

### 10.5.1. Phage-Resistant Starters

Bacteriophage infections represent a serious problem in dairy fermentative processes, and their inhibitory effect against lactic acid starters has been recognized for more than 70 years (Moineau and Lévesque 2005), since Whitehead and Cox (1935) identified the first phage specific for a lactic acid bacterium. Mutation and recombination with other lytic phages (Brüssow and Hendrix 2002) or with the bacterial host chromosome (Moineau et al. 1994) are nowadays accepted as the cause of the continuous emergence of new phages in dairy environments. Additionally, lysogenic starter cultures (Moineau et al. 1996; Bruttin et al. 1997; Sturino and Klaenhammer 2004; Suárez et al. 2008) and raw milk as a supply of phages capable to survive heat treatments (Quiberoni et al. 1998, 2003; Binetti and Reinheimer 2000; Capra et al. 2004) are the major sources of a permanent and renovate phage population. Thus, considering that the presence of phages is unavoidable in the dairy plant environments, several strategies were designed to control rather than eliminate them (Moineau and Lévesque 2005). Culture rotation programs, direct vat-inoculation of starters, careful handling and disposal of whey (Coffey and Ross 2002), use of phage-inhibitory media, optimized sanitation, and use of starter cultures improved in their phage resistance (Moineau et al. 2002; Sturino and Klaenhammer 2004) could be enumerated among the approaches applied to minimize phage spreading in dairy plants.

Since *L. lactis* and *Strep. thermophilus* constitute the key of most industrial dairy fermentations, multiple phages infecting these two LAB have been isolated and meticulously characterized (Brüssow 2001; Suárez et al. 2002; Sturino and Klaenhammer 2004). The extended co-survival of LAB and phages within the same environment has prompted the strains to the acquisition of a variety of native bacteriophage defense systems. These mechanisms include inhibition of phage adsorption, blocking of DNA injection, restriction/modification systems, and abortive infection (Moineau and Lévesque 2005). In lactococci, these mechanisms may be encoded by chromosomal or plasmid genes. Interestingly, the natural gene transfer by conjugation of plasmid DNA is a very spread feature in lactococci. So, the conjugation of native phage-resistant plasmids has been a strategy of great profit to genetically improve dairy LAB for over 20 years, yielding multiple dairy starter cultures that are in commercial use since many years ago, many of them being under patent worldwide (Klaenhammer and Fitzgerald 1994; Moineau and Lévesque 2005). However, although the conjugal transfer of phage resistance plasmids represents nowadays one of the most convenient, simple, and “natural” strategy to improve starter strains, it can be severely limited due to the lack of suitable selectable markers on the plasmids. In this respect, Mills et al. (2002) have exploited lactacin 481 as a selectable marker for the transfer of a phage resistance plasmid into a cheese starter *L. lactis* strain. This approach has a double advantage as the new developed transconjugants were phage-resistant and bacteriocin producers, this last aspect being beneficial also for industrial fermentations.

The isolation of bacteriophage-insensitive mutants (BIMs) represents an alternative for bacteria without conjugative plasmids. These variants are spontaneous and natural derivatives that survive to an exposure to virulent phages (Moineau and Lévesque 2005). In this sense, several researches have described the isolation of spontaneous phage-resistant variants from sensitive strains of lactococci (Limsowtin and Terzaghi 1976; Weimer et al. 1993), *Strep. thermophilus* (Viscardi et al. 2003; Binetti

et al. 2007), *Lact. helveticus* (Neviani et al. 1992; Carminati et al. 1993; Reinheimer et al. 1993; Quiberoni et al. 1999), and *Lact. delbrueckii* (Guglielmotti et al. 2006) strains. This methodology has been revalued recently because it is simple and “natural” and involves no genetic manipulation, thus there are no regulatory restrictions to use the improved strains in industrial environments (Sing and Klaenhammer 1993; Weimer et al. 1993; Moineau and Lévesque 2005). Even if some disadvantages (e.g., a high frequency of phenotype reversion and physiological bacterial modifications) could be encountered when applying this methodology, many phage-resistant variants with high technological performance have been isolated and are being used successfully in industrial fermentations (Quiberoni et al. 1999).

The mechanism involved in BIMs generation was often attributed to mutations in the phage receptors (Viscardi et al. 2003), although recent studies (Barrangou et al. 2007; Deveau et al. 2008) have demonstrated that clustered regularly interspaced short palindromic repeats (CRISPRs) play a role in the development of BIMs. By using *Strep. thermophilus* strains, the authors proved that CRISPRs and the associated genes constitute the latest defense mechanism unveiled in prokaryotes. Specifically, Deveau et al. (2008) found that in response to challenges with virulent phages, a *Strep. thermophilus* strain was able to integrate new spacers derived from the phage genomes, generating a phage-resistant phenotype; meanwhile a small population of phages was also able to infect the BIMs, suggesting that both CRISPR locus and phage genomics regions may rapidly evolve. As stated by Deveau et al. (2008), these facts clearly represent a novel and interesting approach for the development of phage-resistant bacterial strains for fermentation and biotechnological processes.

If the development or use of transconjugants or phage-resistant mutants is not feasible in any cases, the construction of genetically engineered strains is nowadays possible, since it has been intensively studied for the last 25 years. The designed genetic tools are based on the characterization and exploitation of the LAB native phage defense mechanisms,

as well as some phage genetic elements (Moineau 1999; Sturino and Klaenhammer 2004). These type of antiphage approaches include origin-derived phage-encoded resistance (O’Sullivan et al. 1993), antisense RNA technology (interfering with phage development by inhibiting the translation of phage-encoded genes necessary for normal development; Walker and Klaenhammer 2000), and phage-triggered suicide systems (involving the expression of a toxic gene product under the control of a phage-inducible promoter; Djordjevic et al. 1997). Nevertheless, despite the intensive research and economic efforts, dairy and starter culture industries have not benefited as expected (Sturino and Klaenhammer 2004). This is mainly due to the lack of progress in the development of legislation regarding Genetically Modified Organisms (see Chapter 20). Moreover, much of the research has been done using a small subset of lactococcal strains and not the wide range of industrial LAB strains (Coffey and Ross 2002).

The scenery of phage infections is even worse when probiotic bacteria are the target. The manufacture of certain types of probiotic products involves a propagation of the strains as a starter (Watanabe et al. 1970; Forsman et al. 1993; Saarela et al. 2000; Capra et al. 2006). These strains usually grow slowly (Saarela et al. 2000), thus becoming particularly vulnerable to phages (Capra et al. 2006). Also, *Lactobacillus* strains have long been known to harbor prophages, yielding to the possibility of spontaneous prophage induction during use, or of prophage DNA involvement in the generation of new virulent phages (Durmaz et al. 2008). The dairy industry must deal with a new complex and singular trouble. For probiotic phages, control strategies are restricted because these phages are resistant to thermal and biocide treatments (Capra et al. 2004); strain rotation is inappropriate; and, also, many phages originated from lysogenic probiotic strains (Ventura et al. 2006; Durmaz et al. 2008). Furthermore, the development of phage-resistant probiotic starters is very limited, as the preservation of probiotic features is still a non-explored but crucial issue to be considered in practice.



### 10.5.2. Starter Adjuncts

Starter adjuncts are selected cultures added for purposes other than acid formation, which is exclusively devoted to the primary LAB starter. Starter adjuncts can be used as ripening cultures, for example, as cultures added to accelerate ripening or produce desirable flavor, or they can also contribute to the microbial safety or offer health benefits. Several pools of nonstarter microorganisms are now available for the cheese maker to choose from for the cheese innovation. Among them, nonstarter LAB (NSLAB) form a significant portion of the nonstarter microflora of most cheese varieties during ripening and have also been suggested as microbial fingerprint for traditional cheeses. NSLAB are adventitious microorganisms that develop in nearly all cheese varieties and consist mostly of facultative heterofermentative (mesophilic) lactobacilli (FHL); *Lact. casei*, *Lact. paracasei*, *Lact. plantarum*, *Lactobacillus rhamnosus*, and *Lactobacillus curvatus* are found most frequently in cheese (Chamba and Irlinger 2004).

**Ripening cultures.** The significance of FHL to cheese quality is controversial: while some authors have postulated that they are responsible for most quality defects in cheese (Herreros et al. 2007; Rynne et al. 2007), other researchers reported that FHL do not alter flavor development (Lloyd et al. 1980), or even that they can positively influence secondary proteolysis and flavor (Hynes et al. 2003; Di Cagno et al. 2006; Irigoyen et al. 2007). In addition, adjunct of FHL may suppress the growth of undesirable adventitious microbiota and thereby prevent the occurrence of defects (Banks and Williams 2004). Adjunct cultures of cheese-isolated FHL have therefore been proposed as ripening cultures, based on their contribution to secondary proteolysis and flavor formation in Cheddar (McSweeney et al. 1994), Danbo (Antonsson et al. 2003), Manchego (Poveda et al. 2003), and Armada cheeses (Herreros et al. 2007) among others, but the indications appear rather controversial. The main reason for this statement is that FHL exhibit a large diversity of properties, which offer several ways for

their selection as adjuncts. These properties, however, are strongly strain-dependant and they make it difficult to select the right strain combination that links the required properties to the concomitant lack of imperfections.

The peptidase activities, at least in certain *Lact. casei* strains, contribute to the hydrolysis of bitter peptides to non-bitter peptides with the release of free amino acids (Chamba and Irlinger 2004). Amino acid catabolism and the production of aroma compounds by FHL, especially by their glutamate dehydrogenase (GDH) activity, appear to be determinant properties for use of these lactobacilli as cheese ripening adjuncts. Tanous et al. (2002) showed that about half of the *Lact. plantarum* and *Lact. paracasei* strains isolated from cheese have GDH activity. Unfortunately, comparative studies on the behavior of a given strain in different cheese models are lacking; these studies can, however, provide interesting information, as cheese-making conditions (i.e., cheese technology, cooling rate, ripening temperature, etc.) and primary starter may favor or impair the growth of the adjunct culture and strongly influence on its enzymatic activities. The finding of versatile cultures, with desirable technological properties robust enough to be expressed in different food environments and in the presence of different starter cultures, may be useful for the cheese industry and its suppliers.

**Protective cultures.** Protective cultures are starter adjuncts showing specific antimicrobial activity against pathogenic or spoilage bacteria. LAB produce several natural antimicrobials, including organic acids, carbon dioxide, hydrogen peroxide, diacetyl, ethanol, and antimicrobial proteins (or bacteriocins) and, for this reason, they may help to combat microbial contamination. Both starter LAB and NSLAB can be used as protective cultures. Their application in cheese will depend on the target microorganism(s), the product type, and the spectrum of activity of the antimicrobial produced.

Within the wide range of antimicrobials produced by LAB, bacteriocins are the most widely studied and considered for an application in cheese. Bacteriocins from LAB are low-molecular mass



peptides or proteins with an antibacterial mode of action restricted to related Gram (+) bacteria. Although nisin produced by *L. lactis* is the most widely studied and applied bacteriocin, several other LAB bacteriocins (especially those produced by *Strep. thermophilus*, *Strep. macedonicus*, and enterococci) with potential use in cheese have been reported (Grattepanche et al. 2008). Several studies have demonstrated the potential of bacteriocins to control growth of pathogenic or spoilage bacteria in cheese. Most research of LAB bacteriocins in cheese have targeted the control of *Listeria monocytogenes* and the prevention of late blowing of cheese due to contamination by clostridia (Leroy and De Vuyst 2004; Grattepanche et al. 2008). Another example is the suppression of flavor-disturbing *L. lactis* strains that produce off-flavors in dairy products (Stanley 1998). Strains of *Lact. curvatus*, *Lact. plantarum*, and *Lact. rhamnosus* produce bacteriocins active against clostridia, *Staphylococcus aureus*, and *Listeria* spp. (Chamba and Irlinger 2004).

The production of inhibitory compounds and bacteriocins can also be problematic in cheese. For example, bacteriocin-producing strains included within the primary starter may inhibit the other starter components, leading to strain imbalance of the culture. *Lact. casei* and *Lact. rhamnosus* producing acetate, formate, and small amounts of diacetyl from citrate interfere negatively with the growth of *Propionibacterium freudenreichii* in Swiss cheese (Chamba and Irlinger 2004). Additionally, the use of bacteriocin-producing strains may raise safety issues, such as in the case of enterococci. Moreover, bacteriocin production and efficiency can greatly vary within cheese. Bacteriocin-producing strains used as starter adjuncts must also show good growth in milk and cheese. Therefore, the interest of bacteriocinogenic strains as protective cultures is very variable and depending on several technological and ecological factors, which may partly explain why few applications have reached the market. Selection and isolation of competitive bacteriocinogenic strains from cheese environments and design of proper starters with low sensitivity to the bacteriocin will be basic prerequisites for their successful large-scale application as protective cultures.

### 10.5.3. Probiotic/Health-Promoting Cultures

The “probiotic” concept has evolved from the beginning to an actual, simple, and straightforward notion: live microorganisms that when administered in adequate amounts confer a health benefit on the host (FAO/WHO 2002). Consequently, a wide variety of genera and species could be considered potential probiotics even when, commercially, the most important strains are lactobacilli and bifidobacteria (Vasiljevic and Shah 2008). A number of health benefits have been attributed to products containing probiotic organisms, including alleviation of lactose intolerance, prevention and reduction of symptoms of rotavirus and antibiotic-associated diarrhea, treatment and prevention of allergy, reduction of risk associated with mutagenicity and carcinogenicity, hypocholesterolemic effect, inhibition of *Helicobacter pylori* and intestinal pathogens, prevention of inflammatory bowel diseases, and stimulation of immune system (Vasiljevic and Shah 2008).

In selecting starter microorganisms, reliable acid-forming ability is one of the most relevant characteristics to be taken in consideration. However, for a careful screening of probiotics, this criterion should be connected to the impact on human health and well-being. Probiotics are often inappropriate as starter organisms since the gastrointestinal tract environment (the usual ambient of probiotics) is quite different from that of a food matrix. In order to improve the suitability of the food as a substrate for probiotics, energy sources (e.g., glucose), growth factors (e.g., yeast extract and protein hydrolysates), or suitable antioxidants, minerals, or vitamins can be added. Moreover, several technological aspects, including good sensory properties, phage resistance, viability during processing, and stability both in the product and during storage, must to be considered when using probiotic strains (Mattila-Sandholm et al. 2002).

In general, fermentation technologies can significantly affect the functionality of probiotics (Mattila-Sandholm et al. 2002). To avoid the stressful conditions involved in industrial processes,

probiotics are used, in most of cases, as adjunct starters at the critical concentration to guarantee their functionality (at least,  $10^7$  cfu/g or ml, according to international consensus). However, some strains (specially belonging to the *Lact. casei* Group and *Lact. plantarum*) are able to grow on lactic- and other substrates, and exert the role of probiotic starters. In these cases, besides its functional benefit, a technological function would be developed by probiotics, representing a valid strategy to overcome the barriers imposed by the drastic change of environmental conditions. In addition to modifications introduced in the food matrix, a considerable reduction in production costs would be achieved, since lower initial concentrations of probiotic strains are required.

*Lact. plantarum* is a versatile lactic acid bacterium that can be found in a wide range of environmental niches. In consequence, it can be used as starter in dairy, meat and a large variety of vegetable fermentations. It is commonly found in the human gastrointestinal-tract and, therefore, has a demonstrated safety for human consumption and a proven ability to exert a probiotic effect on the consumer (de Vries et al. 2006). Although most applications of *Lact. plantarum* involve the fermentation of vegetal substrates (brined olives, sauerkraut, salted gherkins, sourdough, Nigerian ogi, Ethiopian kocho, Gari, the functional beverage commercially named ProViva, etc.) (de Vries et al. 2006; Molin 2008), this species appears as starter component (combined with other microorganisms) in a high number of traditional dairy products as cheeses and fermented milks (Ercolini et al. 2003; Patrignani et al. 2006; De Angelis et al. 2008; Duan et al. 2008; Mangia et al. 2008; Mathara et al. 2008; Rantsiou et al. 2008). It is important to highlight that several *Lact. plantarum* strains have been tested for functional effects on healthy and unwell subjects (de Vries et al. 2006; Klarin et al. 2008; Szymanski et al. 2008; Troost et al. 2008). For these reasons, *Lact. plantarum* emerges as a species of great interest for the development of new functional foods, based on its probiotic potential as well as on their adaptability and versatility in different substrates that allow it to assume the starter role.

Other microorganisms with demonstrated probiotic properties and ability to grow on dairy substrates are species belonging to the *Lact. casei* Group (*Lact. casei*, *Lact. paracasei* and *Lact. rhamnosus*). The probiotic strains *Lact. casei* Shirota (LcS) and *Lact. casei* DN-114 001 are involved in the elaboration of Yakult and Actimel, respectively, the fermented milks of highest remarkable commercial relevance in the world. In both cases, the industrial processes include several days of strictly controlled fermentation. Numerous health properties have been well documented for these fermented products (de Moreno et al. 2008; Giralt et al. 2008; Miyazaki and Matsuzaki 2008).

Bertazzoni Minelli et al. (2004) evaluated the technological performance of *Lact. casei* strains by determining their suitability as probiotic agents in functional fermented milks when growing in fiber-rich milk (milk supplemented with milk proteins, sucrose and inulin). Several strains, potentially probiotics, showed adequate technological and organoleptic characteristics for production of fermented milks, since an acceptable acidification capacity (pH reduction to 4.3 in 9 h at 37°C) was verified. Moreover, a final product with good texture and without off-flavors was obtained, indicating the potential use of these probiotic *Lact. casei* strains as starter.

Another type of fermented milk involving probiotic species is the acidophilus milk, the so-called "yogurt mild," which became a success in Germany and some other Western European countries in the early 1980s. This product is fermented by a thermophilic culture of *Strep. thermophilus* combined with *Lact. acidophilus*, requiring prolonged periods of fermentation (>6 h) at 38–45°C to guarantee the formation of a stable gel from coagulated milk (pH < 4.8) (Heller 2001). Recently, Amiri et al. (2008) studied a symbiotic acidophilus milk with satisfactory functional food properties. In this case, three different probiotic cultures were employed in combination (*Lact. acidophilus*, *Lact. casei*, and *Bifidobacterium bifidum*) and the incorporation of inulin, oat fiber, and honey, singly or combined resulted in an acceptable probiotic acidophilus milk. In any case, as well as in all fermentation processes

involving probiotic strains, a compromise between full expression of the potential health properties of the probiotic strain and its technological suitability must be reached. The probiotic strain must satisfy not only the criteria for good survival but also the criteria for fermentation and harmonious interaction with the other bacterial species involved. In consequence, the *Lact. acidophilus* strain with the optimum combination of functional and technological properties would represent the best candidate to include in the elaboration of a probiotic acidophilus milk.

More recently, Almeida et al. (2008) investigated the acidification rates of different probiotic strains (*Lact. delbrueckii* subsp. *bulgaricus*, *Lact. acidophilus*, *Lact. rhamnosus*, and *B. animalis* subsp. *lactis*) in co-culture with *Strep. thermophilus* for the production of a probiotic beverage from a Brazilian cheese whey. In general, the fermentation times were very variable (from 3 to 12 h) depending on the co-culture. The longest fermentation time was observed for *Lact. rhamnosus*–*Strep. thermophilus* mixture, whereas the faster acidification was achieved by *B. animalis* subsp. *lactis*–*Strep. thermophilus*. Although more studies are required on wider mix of co-cultures, the innovation of this work lies on the study of technological performances of probiotics, addressed to satisfy the increasing request of the market.

Cheese represents a very attractive food matrix for the incorporation of probiotic bacteria, usually as adjuncts. Particularly, in the Argentinian probiotic Fresco cheese, the *Lact. casei* strain used as adjunct starter was able to grow (from 1 to 1.5 log) through the ripening period and the refrigerated storage (Vinderola et al. 2000). In this context, this probiotic strain could act as a secondary starter to control NSLAB adventitious flora besides as being a functional culture. Similarly, other lactobacilli (mainly *Lact. plantarum*, *Lactobacillus fermentum* and *Lact. rhamnosus*) may be used in cheeses processes for this double purpose: secondary- and probiotic starter. In general, these species acidify slowly in milk and show weak to moderate proteolytic activity, but they may be able to attempt high counts in cheese during ripening as a result of their pepti-

dolytic activity. As a consequence, cheeses containing adjunct probiotic lactobacilli show improved flavor intensity, acceptability and high levels of free amino acids (Bude Ugarte et al. 2006; Briggiler Marcó et al. 2007; Milesi et al. 2008; Kocaoglu-Vurma et al. 2008) in addition to the resulting health benefits.

In most of mentioned examples, probiotic strains are combined with yogurt starter cultures for allowing the production of fermented products with superior sensorial and nutritional properties. The basic components of traditional yogurt, *Strep. thermophilus* and *Lact. delbrueckii* subsp. *bulgaricus* are not usually part of the indigenous microbiota of mammal intestine and show, in general, limited survival after oral ingestion (Bertazzoni Minelli et al. 2004). However, lactic acid starter species (*Strep. thermophilus*, *Lact. delbrueckii* or *Lact. helveticus*) are now also regarded as microorganisms with probiotic potential. Recent studies have shown that these organisms can release enzymes such as  $\beta$ -galactosidase that improve the digestion of nutrients in the intestine and contribute to the normal development of the gut mucosa immune system (de Moreno and Perdígón 2005; Guarner et al. 2005). The potential probiotic role of *Lact. delbrueckii* has not yet been extensively exploited and further studies are needed to identify the beneficial effects on host health of individual strains. Nevertheless, recently, the ability to activate the gut mucosal immune response (specifically on IgA-producing cells) was reported for different *Lact. delbrueckii* strains and their phage-resistant spontaneous mutants (Guglielmotti et al. 2007a; Vinderola et al. 2007). Acquisition of oxgall-tolerance and adequate technological properties were also reported by these strains (Guglielmotti et al. 2006, 2007b). All these features, added to their improved phage resistance, highlight the potential of these strains as probiotic starters for industrial use in functional food.

In conclusion, even though the most important characteristic of probiotic bacteria is their contribution to host health, the evaluation of their technological behavior should be a key parameter for the rational selection of adequate strains for food applications. For a starter LAB, determination of

its technological performance should precede investigation of its probiotic potential. However, if the organism exhibits desirable properties of both types, it would be attractive for use in dairy products as probiotic starter. In particular, the innovations requiring novel types of starter cultures are dependent on the delicate balance among factors such as science and technology, safety and legislation, market needs, consumer attitudes, and economics.

## 10.6. Future Trends and Final Considerations

Since raw milk and natural cultures contain LAB strains which are essential for producing the desired characteristics of cheeses, the study of their microbiota remains a major subject to gain insight on the metabolic and physiological properties of the starter cultures. The selection of target strains with desired traits from this huge pool of LAB by traditional methods, results in a difficult and cumbersome task. In this regard, whole-genome analysis using bioinformatics tools should expand our knowledge on metabolic pathways and mechanisms in different LAB. Bioinformatics could be applied to search in genomes for specific genes, gene clusters, or functionalities in view of the availability of genome sequences of several LAB (Klaenhammer et al. 2005). Bioinformatics can be used to search in genomes for essential components involved in cheese ripening, such as proteinases, peptidases, aminotransferases, enzymes for biosynthesis of amino acids, and transport systems for peptides and aminoacids (van Kranenburg et al. 2002). With the recent development of microarray technology and comparative genomics, wide strain collections would be rapidly and efficiently screened for the presence of certain desired traits. The selection of new starter strains with an added value would thus be facilitated enormously (Liu et al. 2008). Genomics (such as DNA microarrays) and high-throughput screening approaches (e.g. GC-MS analysis of flavor profiles of fermentation fluids) allow the targeted selection of, for example, flavor-forming cultures in dairy fermentation.

However, fundamental and applied research is still needed to better improve starter cultures in the existing production technology and to obtain quantitative data which may yield precious information about the relationship between the cheese environment and bacterial functionality, thus contributing to optimal strain selection. Quality, safety, and acceptability of both traditional and industrial fermented dairy products may be significantly improved through the use of starter cultures implemented on a multifunctional basis (e.g., efficient acid and aroma production, overproduction of bacteriocins, effective proteolytic systems) taking also into consideration the probiotic aspects and health-promoting properties.

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## Chapter 11

# Low-Calorie Sugars Produced by Lactic Acid Bacteria

Gino Vrancken, Tom Rimaux, Luc De Vuyst, and Fernanda Mozzi

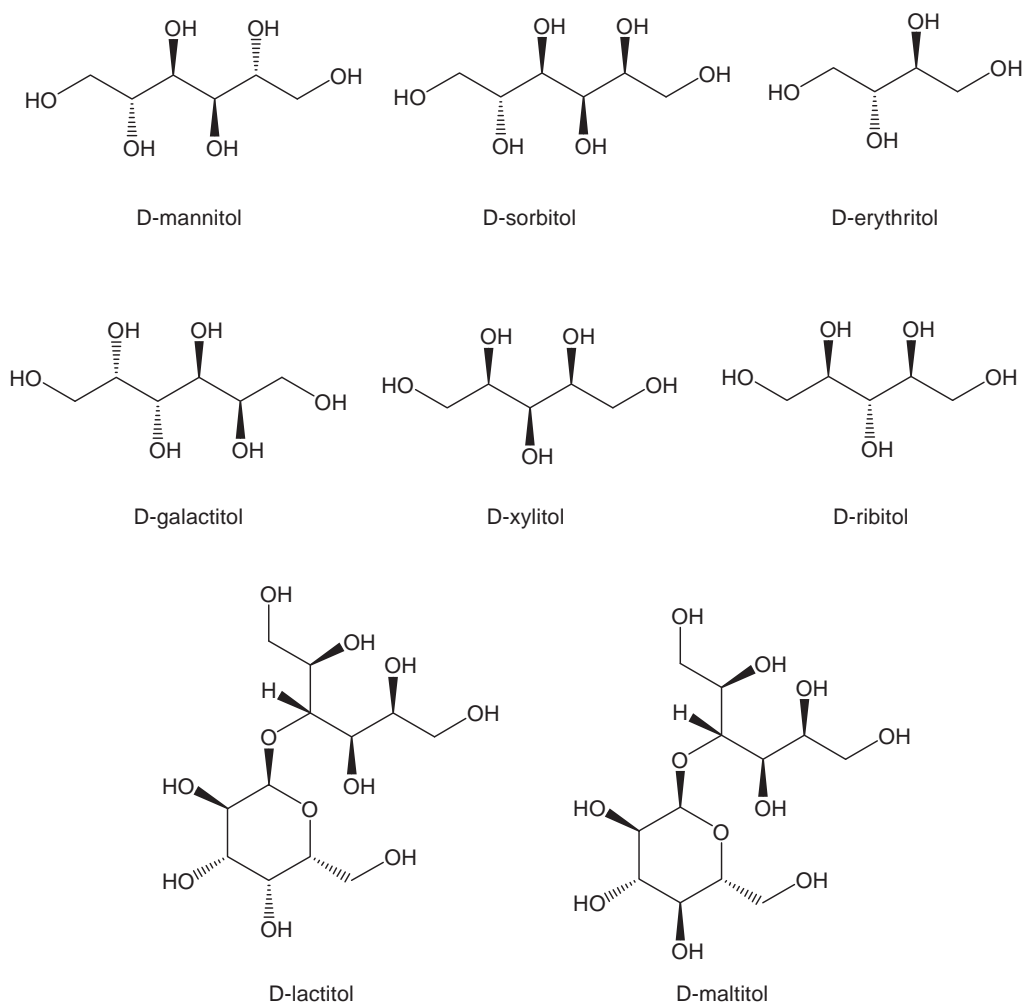
*Mannitol, sorbitol, and erythritol are naturally occurring sugar alcohols. Mannitol is produced by bacteria, yeasts, fungi, algae, and several plants. This polyol might help these organisms to cope with different environmental stresses such as osmotic and oxidative stress. Sorbitol is produced by a variety of both plants and microorganisms. Erythritol production is usually associated with yeasts but has also been reported for some lactic acid bacteria (LAB). All these polyols—mannitol, sorbitol, and erythritol—display properties that are beneficial to human health as they are non-metabolizable, insulin-independent sweeteners, or low-calorie sugars, which make them applicable in dietetic and diabetic food products. In addition, mannitol is used in the pharmaceutical industry as a powerful osmotic diuretic agent and as an osmotic agent for decreasing brain and cellular edema. Mannitol biosynthesis through bacterial fermentation has become an interesting alternative to existing chemical production. Chemical synthesis using nickel catalyst-assisted hydrogenation shows several economic disadvantages, such as high production costs and low product recovery. Furthermore, the capability of certain LAB, belonging to both homofermentative and heterofermentative species, to synthesize mannitol offers the possibility of in situ production in foods. For this reason, different fermentation technology-based strategies for improving mannitol production by LAB have been reported. To date, 93–97 mol% mannitol yields are reached using a bioprocess with a heterofermentative LAB strain. Moreover, metabolic engineering of food-grade lactate dehydroge-*

*nase-deficient mutants has been reported, in particular for sorbitol production by LAB. This chapter deals with the state of the art of sugar alcohol (mannitol, sorbitol, and erythritol) production by LAB as interesting low-calorie food ingredients. In addition, the strategies employed to increase the production and application of sugar alcohols in food and pharmaceutical products, or to design new functional foods naturally enriched in mannitol will be discussed.*

### 11.1. Classification and Natural Occurrence

Sugar alcohols are noncyclic, polyhydric alcohols (polyols), in which the carbonyl group (aldehyde or ketone) of the precursor sugar is reduced to the corresponding primary or secondary alcohol (Schiweck et al. 1994; Cummings and Stephen 2007). Due to different structures and stereochemistries of the precursor sugars, several distinct polyols exist, such as mannitol (derived from fructose), sorbitol (from glucose), galactitol (from galactose), xylitol (from xylose), ribitol (from ribose), lactitol (from lactose), maltitol (from maltose), and erythritol (from erythrose) (Fig. 11.1).

Polyols occur naturally in fruits and vegetables and are synthesized by certain bacteria, yeasts, filamentous fungi, and algae (Mäkinen and Soderlinge 1980; Hendriksen et al. 1988; Stoop et al., 1996; von Weymarn et al. 2002a; Wisselink et al. 2002; Iwamoto and Shiraiwa 2005; Solomon et al. 2007). Their physiological role remains a subject of debate,



**Figure 11.1** Chemical structures of D-mannitol, D-sorbitol, D-erythritol, D-galactitol, D-xylitol, D-ribitol, D-lactitol, and D-maltitol.

but they have been proposed as reservoirs of carbon and reducing power. A role has also been suggested in surviving adverse environmental conditions such as osmotic and oxidative stress, as compatible solute (turgor maintenance, and membrane lipid and protein stabilization at low water activity), and in preventing oxidative damage by scavenging of free reactive oxygen radicals (Smirnov and Cumbe 1989; Chaturvedi et al. 1997; Shen et al. 1997; Efiuvwevwe et al. 1999; Wisselink et al. 2002;

Iwamoto and Shiraiwa 2005; Akinterinwa et al. 2008; Hounsou et al. 2008).

D-mannitol, also called mannite or manna sugar, the acyclic hexitol derived from D-fructose, is the most abundant naturally occurring polyol, even more extensively than its C2-epimer sorbitol. Mannitol is found in manna, the dried exudates of the manna ash tree, *Fraxinus ornus*, from which its name derives (Schwarz 1994). In general, mannitol occurs in small quantities in most fruits and vegeta-



bles, such as pumpkins, celery, onions, olives, and beets. Mistletoe and lichen mycobionts and some marine algae, such as brown seaweeds, are also rich in mannitol (Brimacombe and Webber 1972; Ikawa et al. 1972; Fahselt 1994; Schwarz 1994; Stoop et al. 1996). Mannitol is commonly found in the mycelium of various fungi and is an important carbohydrate in the commonly consumed fruiting bodies of the fungi *Agaricus bisporus* and *Lentinula edodes* (Tan and Moore 1994). The optical isomer L-mannitol does not occur in nature. This chapter will specifically deal with the D-form of mannitol, which will be further referred to here as mannitol.

D-sorbitol, also called D-glucitol, the noncyclic hexitol derived from D-glucose, occurs naturally in many fruits, such as berries, cherries, grapes, plums, pears, and apples, as well as in certain vegetables (Budavari et al. 1996; Gutierrez and Gaudillere 1996). In addition, various yeasts and bacteria are able to synthesize sorbitol (Silveira and Jonas 2002; Jonas and Silveira 2004).

D-erythritol, the four-carbon sugar alcohol derived from the sugar erythrose, is naturally present in fruits such as grapes, pears, and melons, but also occurs in mushrooms and some fermented food products, such as soy sauce, sake, and wine (Embuscado and Patil 2001).

Mannitol, sorbitol, and erythritol have all received Generally Recognized as Safe (GRAS) status by the U.S. Food and Drug Administration (Salminen and Hallikainen 2002). The international world market for polyols is primarily for sorbitol. Mannitol and erythritol, introduced in the market in the 1990s, represent a lower production volume than sorbitol (von Weymarn et al. 2002a).

## 11.2. Physicochemical Properties and Applications of Mannitol, Sorbitol, and Erythritol

Polyols are known mainly for their low-calorie sweetening power (Salminen and Hallikainen 2002). This sweetening power varies among the different sugar alcohols: The relative sweetness of mannitol to sucrose is 50%–52%, that of sorbitol to sucrose is 60%–70%, and that of erythritol to sucrose is

53%–70% (Schiweck et al. 1994; Embuscado and Patil 2001; Salminen and Hallikainen 2002). Mannitol forms white crystals with a melting temperature of 165–168°C, and its solubility in water (18% [m/v] at 25°C) is significantly lower (fourfold) than that of sorbitol and most of the other sugar alcohols (Schwarz 1994). The high positive enthalpy of solution in water gives mannitol a sweet cool taste (Schiweck et al. 1994; Lawson 1997). Erythritol has the highest enthalpy of solution (191.3 J/kg) of the three sugar alcohols described here, thus providing a substantially higher cooling effect (Embuscado and Patil 2001). Crystalline mannitol and erythritol exhibit a very low hygroscopicity and are chemically inert (Schwarz 1994).

Because of its physicochemical characteristics, mannitol has multiple applications in the food, pharmaceutical, medical, and chemical industries. Mannitol's primary application is as a food additive, referred to as E421 in the European Union (Le and Mulderrig 2001). It is used in dietary food products such as sugar-free (non-cariogenic) chewing gum, sweets, and ice cream (Le and Mulderrig 2001). In addition, it can be used as a texturizing agent, anti-caking agent, or humectant (Salminen and Hallikainen 2002). Mannitol is used to reduce the crystallization tendency of sugars, thereby increasing the physicochemical shelf life of food products. As crystalline, mannitol displays very low hygroscopicity and chemical reactivity as well as excellent thermal and mechanical properties. It is used for preparing pharmaceutical products, such as chewable tablets and granulated powders, at high humidities (Gombas et al. 2003; Sugimoto et al. 2006). Moreover, its sweet cool taste masks the unpleasant taste of certain drugs (Debord et al. 1987).

Sorbitol's major application is as a food additive (E420), especially in confectionery products (chewing gums, candies, desserts, and ice creams), diabetic foods, and a wide range of other food products, not only as a sweetener but also as a humectant, texturizer, and softener. Due to its water stabilization and softening properties, sorbitol is used in the cosmetic industry in creams, emulsions, and lotions (Le and Mulderrig 2001; Silveira and Jonas 2002). Both sorbitol and mannitol have a stabilizing effect

on food by partially mimicking fat (Debord et al. 1987). Another interesting property of sorbitol is that although it is hygroscopic, it tends to adsorb or release water very slowly; hence, it can be used in foods to maintain the initial physicochemical characteristics of the fresh food product (Le and Mulderrig 2001). Finally, sorbitol is the starting material or building block for the synthesis of ascorbic acid (vitamin C through the Reichstein process), sorbose, and alkyd resins, among others (Reuter et al. 1983; Silveira and Jonas 2002; Adachi et al. 2003).

In medicine, mannitol has found application as a powerful osmotic diuretic for intoxication therapy. It reduces cerebral edema during brain surgery, and it is also used in intraocular pressure reduction. In addition, it is employed in parenteral solutions during surgery to prevent kidney failure (Nissenson et al. 1979; Warren and Blantz 1981; Rabinstein 2006). Mannitol hexanitrate can be used as vasodilator in hypertension treatments (Carere-Comes et al. 1952). In the chemical industry, mannitol is used in the production of dry electrolytic capacitors as a complex with boric acid, and in the production of resins and surfactants (Dapo and Stevens 1999; Kitamoto et al. 2002).

Polyols are absorbed more slowly from the gastrointestinal tract than common sugars and have no effect on the blood insulin level. This fact, combined with the sweetening power of these compounds, makes polyols interesting as sweeteners for the food of people with diabetes. Moreover, polyols have a lower caloric value than most of the common sugars (e.g., mannitol has 60% less calories than sucrose). The reduced caloric value of sugar alcohols is a result of the fact that sugar alcohols are only partially absorbed in the small intestine. Thus, a substantial portion of the ingested sugar alcohols reaches the colon, where certain bacteria are able to degrade it (Schiweck et al. 1994). This degradation can lead to the production of several short-chain fatty acids in the colon, such as butyrate, which has been claimed to confer protection against colon cancer (van Munster and Nagengast 1993; Morishita 1994; Liong and Shah 2005). Although these properties make mannitol an interesting candidate for the

elaboration of light or reduced-calorie food products, overconsumption can lead to gastrointestinal discomfort due to gas production, and diarrhea (Schiweck et al. 1994; Le and Mulderrig 2001; Livesey 2003). Mannitol has the lowest laxative threshold observed for sugar alcohols; therefore, it is recommended that daily intake should not exceed 20 g (von Weymarn et al. 2002a).

Sorbitol intake has been shown to significantly increase colonic and cecal butyrate levels in rats, showing the capacity of this polyol to modify the activity of the gut microbiota, thus possibly contributing to healthy colonic mucosa (Sarmiento-Rubiano et al. 2007). In addition, it has been reported that sorbitol can be consumed as an energy source by some species of *Lactobacillus* and *Bifidobacterium* (Rhodes and Kator 1999; Yebra and Pérez-Martínez 2002). For this reason, Farnworth (2001) suggested that sorbitol should be considered a prebiotic.

Erythritol has a similar spectrum of applications to that of the other sugar alcohols. An important advantage of erythritol over the other sugar alcohols is that it has the lowest caloric value (0.2 kcal/g). Furthermore, because of its low molecular mass, it has the unique property of being rapidly absorbed in the small intestine, after which it is excreted through the kidneys, making it unavailable for colonic fermentation. Therefore, its caloric value is further reduced, and its glycemic response is zero. It is suitable for diabetic and tooth-friendly food applications. Like xylitol, erythritol can significantly reduce the caries bacteria *Streptococcus mutans* (Mäkinen et al. 2005). Excessive administration of erythritol poses no threat for the development of intestinal discomfort (Roper and Goossens 1993).

### **11.3. Production of Mannitol, Sorbitol, and Erythritol: Chemical, Enzymatic, and Microbial Processes**

Mannitol production occurs mainly through chemical, enzymatic, or biotechnological processes, as the extraction from plant raw materials is no longer economical (Schwarz 1994). Today, industrial production of mannitol occurs through catalytic hydrogenation of fructose, sucrose, or glucose/fructose

syrops (Schwarz 1994; Ojamo et al. 2000). Worldwide, large-scale production of mannitol is mostly performed in batch instead of continuous processes. The hydrogenation of glucose/fructose mixtures in aqueous solution is catalyzed by a Raney nickel at high temperatures (120–150°C) and high pressures (70–140 atm; Wisniak and Simon 1979; Makkee et al. 1985). The selectivity of the nickel catalyst is such that about half of the fructose ( $\beta$ -fructose) is converted to mannitol and the other half ( $\alpha$ -fructose) to sorbitol, while glucose is exclusively reduced to sorbitol. Thus, the commercial chemical production of mannitol out of fructose is always accompanied by sorbitol production as a side product. In this way, the hydrogenation of a 50/50 fructose/glucose solution leads to a 30/70 mannitol/sorbitol mixture. As the solubility of mannitol is much lower than that of sorbitol, a fractional crystallization process of this mannitol/sorbitol mixture eventually produces pure mannitol (Soetaert et al. 1999).

Mannitol production can be performed enzymatically or biotechnologically through microbial fermentation. In the former case, the reduction of D-fructose to D-mannitol requires an NAD(P) H-dependent mannitol dehydrogenase enzyme (MDH; EC 1.1.1.67). This enzyme has been purified from several microorganisms, such as *Lactobacillus brevis* (Martínez et al. 1963), *Leuconostoc mesenteroides* (Sakai and Yamanaka 1968), *Lactobacillus sanfranciscensis* (Korakli and Vogel 2003), *Saccharomyces cerevisiae* (Quain and Boulton 1987), *Rhodobacter sphaeroides* (Schneider et al. 1993; Schneider and Giffhorn 1994), *Torulaspora delbrueckii* (Nidetzky et al. 1996), *Pseudomonas fluorescens* (Brünker et al. 1997), and the mangrove red alga *Caloglossa leprieurii* (Karsten et al. 1997). The cofactor dependency of MDH is the major limitation of this enzymatic process as NADH and especially NADPH are very expensive, making this enzymatic process economically unfeasible. To counteract this problem, other strategies, such as cofactor regeneration by simultaneous reactions, have been applied. Wichmann et al. (1981) have suggested the simultaneous conversion of fructose and formate, applying MDH and formate dehydro-

genase from a recombinant *Ps. fluorescens*. This process is advantageous for several reasons: Mannitol is the only end-product formed; formate is an inexpensive reagent; and the carbon dioxide released during the cofactor regeneration reaction poses no additional difficulties for downstream processing. The cofactor may also be regenerated in a system where a glucose/fructose mixture is converted into gluconate and mannitol, using glucose dehydrogenase and MDH (Howaldt et al. 1988). In addition, mannose can be enzymatically reduced to mannitol. However, the reversible reaction, mannitol oxidation, is favored over mannose reduction (Stoop et al. 1998). Slatner et al. (1998) described the enzymatic production of pure mannitol from fructose using MDH from *Ps. fluorescens*. Nevertheless, the enzymatic hydrogenation for mannitol production is adversely affected by other factors, such as the retention of cofactors in the reactor with special membranes, the strong product inhibition of MDH, and the high  $K_m$  value of MDH for fructose (Soetaert et al. 1999). Finally, combinations of enzymatic and chemical processes for mannitol production have been performed in which mannitol yield is increased as compared with the sorbitol yield (Moreland et al. 1984; Devos 1993).

Sorbitol production is traditionally carried out through catalytic hydrogenation of D-glucose syrup at a concentration of approximately 50% (m/v). An enzymatic process for the production of sorbitol has also been proposed, based on the glucose-fructose oxidoreductase enzyme of *Zymomonas mobilis*, which allows the simultaneous production of gluconic acid and sorbitol from sucrose (Zachariou and Scopes 1986; Nidetzky et al. 1997; Silva-Martinez et al. 1998). Furthermore, an enzymatic system with cofactor recycling has been proposed based on the enzymes aldose reductase (from *Candida tropicalis*) and glucose dehydrogenase in a charged membrane bioreactor (Ikemi et al. 1990a, 1990b).

Erythritol is mainly produced biotechnologically through a fermentation process with osmophilic yeasts (Embuscado and Patil 2001). Alternatively, erythritol can be chemically produced by reduction of meso-tartaric acid or oxidation of 4,6-O-ethylidene-D-glucose (Embuscado and Patil 2001). Furthermore,

erythritol can be produced by a chemical process where dialdehyde starch is converted into erythritol by a high-temperature chemical reaction in the presence of a nickel catalyst (Pfeifer et al. 1960).

As an interesting alternative to the chemical and enzymatic production, mannitol, sorbitol, and erythritol may be synthesized biotechnologically by microbial fermentation. Biosynthetic routes offer the potential for a safer and environmentally friendly synthesis with enhanced product specificity (Akinterinwa et al. 2008). Certain yeasts (*Saccharomyces cerevisiae*), filamentous fungi (such as *Alternaria alternata* and *Aspergillus nidulans*), and bacteria (*Escherichia coli*, *Bacillus megaterium*, *Corynebacterium glutamicum*, and especially LAB) have been shown to effectively produce mannitol without the cofactor of sorbitol (Itoh et al. 1992; Solomon et al. 2007). Furthermore, these microorganisms have the advantage of regenerating the cofactors needed through sugar catabolism. Based on studies described in the literature, LAB seem to be the most potent mannitol producers (von Weymarn et al. 2002a). Several microorganisms have been suggested as potential sorbitol producers for industrial processes, which include several yeast strains and the ethanol-producing bacterium *Z. mobilis* (Silveira et al. 1999; Silveira and Jonas 2002; Jonas and Silveira 2004). Biotechnological production of erythritol is performed through a yeast fermentation process using osmophilic yeasts belonging to the species *Moniliella*, *Trigonopsis*, *Torulopsis*, and *Candida magnolia* grown on enzymatically hydrolyzed wheat and corn starches (Koh et al. 2003; Park et al. 2005). Although no naturally sorbitol-producing LAB strains are known so far, metabolic engineering of some LAB has been described as an interesting alternative (Ladero et al. 2007).

## 11.4. Food-grade Microorganisms in the Production of Mannitol, Sorbitol, and Erythritol

### 11.4.1. Metabolic Pathways in LAB

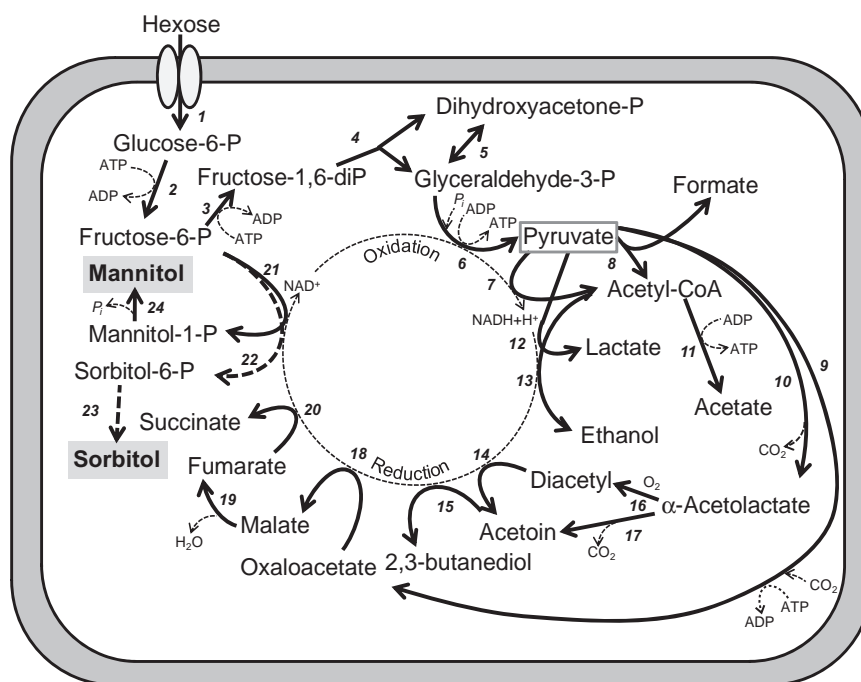
LAB are food-grade (GRAS) microorganisms that are extensively used in the fermented-foods indus-

try, either spontaneously or as starter cultures added to the raw materials, due to their acidifying capacity and contribution to the organoleptic properties of the final food products (Wood 1997). Moreover, several LAB strains displaying health-promoting properties have been used for many years as probiotics in foods and for the production of other functional fermented foods (Stanton et al. 2005). Both classical and functional starter cultures of LAB strains are in use. Classical bulk starter cultures contribute mainly to fast acidification of the raw materials. Functional starter cultures are starters that possess at least one inherent functional property, beyond the production of lactic acid, that can contribute to food safety and/or one or more organoleptic, technological, nutritional, or health advantages (Leroy and De Vuyst 2004). LAB have been shown to be ideal cell factories for the production of several important nutraceuticals through fermentation (Hugenholtz and Smid 2002; Hugenholtz 2008). These compounds include components that have been proven to exert beneficial effects on human health, such as vitamins of the B group and low-calorie sugar alcohols. The biosynthesis of the latter compounds by LAB makes this feature a unique combination of the GRAS characteristic of the polyols and the food-grade status of the producing microorganisms.

Two biosynthetic pathways for mannitol have been described in LAB, depending on the carbohydrate catabolic pathway used: The homolactic and the heterolactic fermentation (Axelsson 2004). In homofermentative LAB, the carbon flow from carbohydrates is directed mainly to lactic acid production using the glycolytic (Embden-Meyerhof-Parnas) pathway (Fig. 11.2); the formation of other fermentation products is only possible when lactic acid production is somehow restricted, thereby creating a necessity for alternative NADH-regenerating pathways. Most homofermentative LAB do not normally produce mannitol, and formation of this sugar alcohol is limited to those strains whose ability to regenerate NAD<sup>+</sup> through lactic acid production is severely hampered. Under such conditions, glycolysis may also lead to a mixed-acid fermentation (Garrigues et al. 1997, 2001; Axelsson 2004), which gives rise to end-products such as acetate, ethanol,

diacetyl, acetoin, 2,3-butanediol, and, in some cases, mannitol (see below). This shift to mixed-acid fermentation is controlled by the glycolytic flux through the level of the enzyme pyruvate–formate lyase (Melchiorsen et al. 2002; Kowalczyk and Bardowski 2007). In these strains, mannitol 1-phosphate dehydrogenase (M1PDH, EC 1.1.1.17), which catalyzes the reversible reduction reaction of fructose 6-phosphate to mannitol 1-phosphate, and mannitol 1-phosphatase (M1Pase, EC 3.1.3.22), and dephosphorylation of mannitol 1-phosphate to mannitol, are the enzymes involved in the mannitol biosynthetic route (Fig. 11.2) (Wisselink et al. 2004). Although the sequence of the gene encoding M1PDH (*mtlD*) has been reported from the complete genome

sequence of the homofermentative strain *Lactococcus lactis* IL-1403 (Bolotin et al. 2001), mannitol production by *L. lactis* and other homofermentative LAB is not very common. Nevertheless, mannitol production by *L. lactis* has been observed by Neves et al. (2000, 2002). In high-density resting-cell suspensions of a lactate dehydrogenase (LDH)-deficient *L. lactis* strain, high levels of mannitol are transiently produced and metabolized once glucose has been depleted. The formation of intracellular mannitol is a consequence of the impairment in NADH oxidation caused by the highly reduced LDH activity of the strain; the transient production of mannitol 1-phosphate serves as a pathway for NAD<sup>+</sup> regeneration. In addition, an LDH-negative mutant of



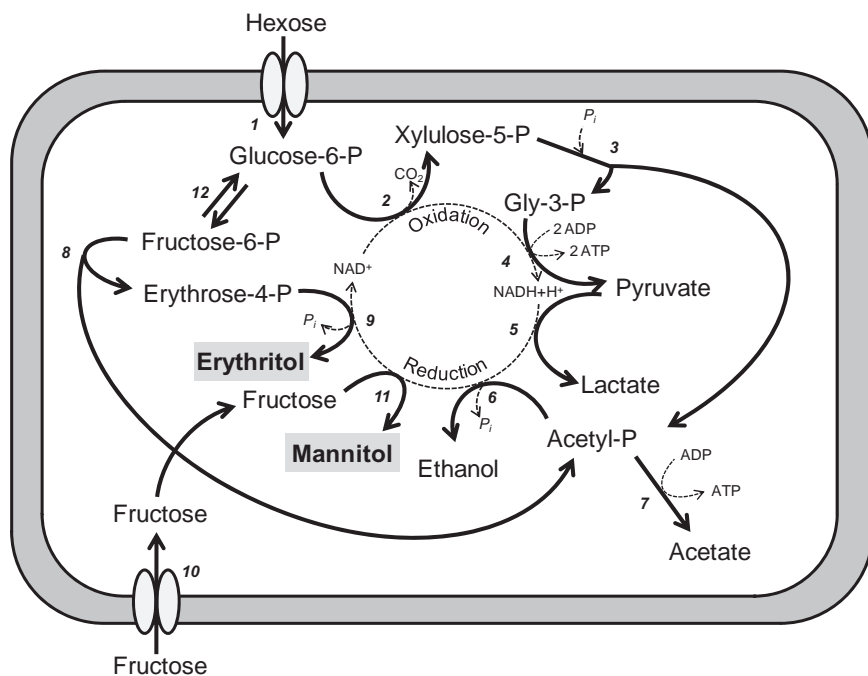
**Figure 11.2** Pathways of hexose metabolism in homofermentative lactic acid bacteria: (1) phosphoenolpyruvate-dependent sugar phosphotransferase system; (2) phosphoglucose isomerase; (3) 6-phosphofructokinase; (4) fructose 1,6-diphosphate aldolase; (5) triose-phosphate isomerase; (6) glyceraldehyde 3-phosphate dehydrogenase, phosphoglycerate kinase, phosphoglyceromutase, enolase, and pyruvate kinase; (7) pyruvate dehydrogenase; (8) pyruvate-formate lyase; (9) pyruvate carboxylase; (10) α-acetolactate synthase; (11) acetate kinase; (12) lactate dehydrogenase; (13) acetaldehyde dehydrogenase and alcohol dehydrogenase; (14) acetoin dehydrogenase; (15) 2,3-butanediol dehydrogenase; (16) chemical oxidation; (17) α-acetolactate decarboxylase; (18) malate dehydrogenase; (19) fumarase; (20) succinate dehydrogenase; (21) mannitol 1-phosphate dehydrogenase; (22) sorbitol 6-phosphate dehydrogenase; (23) sorbitol 6-phosphatase; and (24) mannitol 1-phosphatase. Arrows 22 and 23 indicate genetically engineered steps.



*Lactobacillus plantarum* produces small amounts of mannitol, as reported by Ferain et al. (1996). In these cases, mannitol production is believed to be an alternative pathway to NAD<sup>+</sup> regeneration instead of the usual pathway through lactate formation (Wisselink et al. 2002). Other authors (Chalfan et al. 1975; Loesche and Kornman 1976) have reported mannitol production by strains of *Streptococcus mutans* and *Lactobacillus leichmanii*, albeit at low levels.

By far, the best mannitol-producing LAB strains are those that display heterofermentative metabolism (Fig. 11.3). Heterofermentative LAB use a combination of pathways for carbohydrate catabolism. When the microorganism grows anaerobically on glucose, lactic acid and normally ethanol are produced in equimolar amounts, using the pentose

phosphate (or Warburg–Dickens) pathway. Thus, the microorganism maintains the redox balance by converting acetyl-phosphate into ethanol. If an alternative electron acceptor, such as fructose, is present in the growth medium, the microorganism is capable of using it as such. In the case of fructose, NAD(P)<sup>+</sup> can be regenerated by a fructose-to-mannitol reduction, resulting in acetyl phosphate conversion into acetate instead of ethanol formation, with concomitant ATP production. This supplementary ATP production is the main driving force behind the use of electron acceptors other than acetyl-phosphate (Wisselink et al. 2002; Zaunmüller et al. 2006). Heterofermentative LAB produce mannitol from fructose in a single enzymatic conversion by MDH (Wisselink et al. 2002) without concomitant sorbitol production (Fig. 11.3). Peterson and Fred (1920)



**Figure 11.3** Pathways of hexose metabolism in heterofermentative lactic acid bacteria: (1) phosphoenolpyruvate-dependent sugar phosphotransferase system; (2) glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, and epimerase; (3) phosphoketolase; (4) glyceraldehyde 3-phosphate dehydrogenase, phosphoglycerate kinase, phosphoglyceromutase, enolase, and pyruvate kinase; (5) lactate dehydrogenase; (6) phosphate acetyltransferase, acetaldehyde dehydrogenase, and alcohol dehydrogenase; (7) acetate kinase; (8) phosphoketolase; (9) erythritol dehydrogenase; (10) fructose permease; (11) mannitol dehydrogenase; and (12) glucose-phosphate isomerase.

reported on mannitol production from fructose by a *Lactobacillus* strain. Now, it is well known that species belonging to the genera *Leuconostoc*, *Lactobacillus*, *Streptococcus*, and *Oenococcus* are able to synthesize mannitol (Maicas et al. 2002; von Weymarn 2002a; Korakli and Vogel 2003). In addition, *Leuc. mesenteroides* and *Leuconostoc pseudomesenteroides* produce high levels of mannitol in the presence of fructose or sucrose (Vandamme et al. 1987; Grobbsen et al. 2001). Mannitol has been found in concentrations of up to 150 g/l, which is close to its solubility limit (180 g/l at 25°C), indicating that it has no toxic effects on the organism (Soetaert et al. 1995). Also, Helanto et al. (2005) reported mutants from *Leuc. pseudomesenteroides* obtained by mutagenesis that show high mannitol yields. Two other heterofermentative LAB species, namely *Lactobacillus brevis* and *Lactobacillus buchneri*, are able to produce mannitol from fructose during silage fermentation (Nishino et al. 2003). Saha and Nakamura (2003) found several mannitol-producing LAB strains belonging to different *Lactobacillus* species (*Lact. brevis*, *Lact. buchneri*, *Lactobacillus cellobiosus*, *Lactobacillus fermentum*, *Lactobacillus intermedius*, *Leuconostoc amelibiosum*, *Leuconostoc citrovorum*, *Leuc. mesenteroides* subsp. *dextranicum*, and *Leuc. paramesenteroides*). Finally, *Lact. fermentum* has often been found to produce mannitol during food fermentation processes, such as in cocoa and sourdough fermentations (Camu et al. 2007; Van der Meulen et al. 2007; Vrancken et al. 2008).

In some cases, heterofermentative LAB might also result in the production of erythritol, starting from the intracellular glycolytic intermediate fructose 6-phosphate, which is split by a phosphoketolase enzyme into acetyl phosphate, and erythrose 4-phosphate, which is subsequently reduced to erythritol (Fig. 11.3). Erythritol production has been observed for *Oenococcus oeni*, *Leuc. mesenteroides*, and *Lact. sanfranciscensis* (Veiga-da-Cunha et al. 1992, 1993; Stolz et al. 1995; Richter et al. 2001). LAB do not produce sorbitol naturally. Only a few microorganisms have been suggested as sorbitol producers, such as yeasts and the bacterium *Z. mobilis*; the latter produces sorbitol using the

single enzyme glucose-fructose oxidoreductase (EC 1.1.99.28; Silva-Martinez et al. 1998).

As is the case for the reduction of external fructose to mannitol by MDH in heterofermentative LAB, similar reactions can take place in homofermentative LAB; for instance, fructose 6-phosphate can be reduced to mannitol 1-phosphate and sorbitol 6-phosphate, which are catalyzed by M1PDH, and sorbitol 6-phosphate dehydrogenase (S6PDH), respectively (Nissen et al. 2005). The genes encoding these enzymes are clustered together with the phosphoenolpyruvate-dependent phosphotransferase system of the corresponding hexitol transporter, indicating that their regular physiological role is probably hexitol assimilation instead of hexitol biosynthesis (Bolotin et al. 2001; Yebra and Pérez-Martínez 2002). In *Lactobacillus casei*, a facultative heterofermentative LAB species, two S6PDH-encoding genes (*gutF* and *sorF*), have been found that could make sorbitol production possible. However, sorbitol production by *Lact. casei* does not occur when the species is grown on glucose or lactose since both S6PDH genes are subject to carbon catabolite repression and substrate induction (Yebra et al. 2000; Yebra and Pérez-Martínez 2002). Alternatively, a few *Lactobacillus* strains have been used for producing sorbitol through different metabolic engineering approaches (see below; Nissen et al. 2005; Ladero et al. 2007).

#### 11.4.2. Fermentation Processes to Improve Mannitol Yields

Although several species of LAB have been found to produce mannitol, only for a few strains significant yield improvements have been reported. Efficient mannitol production by heterofermentative LAB is often achieved when NADH is supplied by co-utilization of glucose (von Weymarn et al. 2002a). Diverse fermentation conditions have been investigated to increase mannitol production by LAB. Saha and Nakamura (2003) evaluated the effect of different carbon sources and concentrations during controlled-pH and fed-batch fermentations to improve mannitol biosynthesis by the strain *Lact. intermedius* NRRL B-3693. This strain produced

large amounts of mannitol at high fructose concentrations (300 g/l) as the sole carbon source, reaching values of approximately 200 g/l, which exceeds the mannitol solubility limit of 180 g/l at 25°C. One-third of the fructose concentration may be replaced by other sugars. Korakli et al. (2000) reported that certain LAB can convert fructose almost quantitatively to mannitol when glucose is used as co-substrate. The use of a fed-batch fermentation process allowed a considerable decrease in fermentation time (e.g., from 136 to 92 h) for mannitol production by the strain *Lact. intermedius* NRRL B-3693, although no yield improvements were achieved (Saha and Nakamura 2003). Later, Saha (2006) reported that, by adding manganese sulfate or magnesium sulfate to the growth medium, mannitol production and yield by the same strain are increased. However, mannitol yield increases less with magnesium sulfate than with manganese sulfate. Soetaert et al. (1999) developed and optimized a mannitol fermentation process with a strain of *Leuc. mesenteroides* at constant pH 5.0, 20°C, and slow agitation conditions. The conversion efficiency of fructose into mannitol is over 90% using a mixture of glucose and fructose at a ratio of 1:2; the fermentation is completed after 65 h. Mannitol recovery was carried out from a cell-free fermentation medium by concentration and crystallization at 0–5°C, and a mannitol purity of over 99% was achieved. In addition, Soetaert et al. (1999) reported experiments with continuous mannitol production in a plug-flow reactor with *Leuc. mesenteroides* immobilized on polyurethane foam, leading to a fivefold increase in volumetric productivity, 8 g/l.h, compared with batch fermentations, but the conversion efficiency (60%, as opposed to 76% in batch fermentation) is low.

Resting cells of several heterofermentative LAB strains have been evaluated for their ability to produce mannitol from fructose (von Weymarn et al. 2002b). *Leuc. mesenteroides* ATCC 9135 produced high amounts of mannitol, using high-cell-density membrane cell-recycle cultures. High volumetric mannitol productivity, 26.2 g/l.h, and yield (97 mol%) were achieved. Using the same initial biomass, von Weymarn et al. (2002b) achieved

a stable high-level mannitol production for 14 successive bioconversion batches. Increasing the initial fructose concentration from 100 to 120 g/l and further to 140 g/l resulted in decreased productivities due to both substrate and end-product inhibition of the key enzyme MDH.

Several MDH enzymes have been purified and characterized from different LAB species, such as *Lact. brevis* (Martínez et al. 1963), *Lact. intermedius* (Saha 2004), *Leuc. mesenteroides* (Sakai and Yamanaka 1968), and *Lact. sanfranciscensis* (Korakli and Vogel 2003). *Lact. sanfranciscensis* LTH2590, a strain belonging to the microbiota of traditionally prepared wheat and rye sourdoughs, displayed mannitol biosynthesis (up to 60 g/l) from fructose, using a fed-batch process in a simple bran extract medium, supplemented with a commercial fructose/glucose mixture (Korakli et al. 2000). The production of mannitol by an enzyme purified from *Lact. sanfranciscensis* could be a significant improvement over the chemical production method. The MDH isolated from *Lact. sanfranciscensis* TMW1.392 (isogenic to the LTH2590 strain) has been purified and characterized (Korakli and Vogel 2003). In addition, Saha (2004) isolated and purified the MDH from *Lact. intermedius* NRRL B-3693; this enzyme showed a high affinity for NADPH for reduction of fructose, displayed a very specific substrate specificity, and was able to convert fructose to mannitol with a 100% yield. These results suggest that the isolated enzyme shows potential for use in the cell-free biosynthesis of mannitol from fructose.

#### 11.4.3. Scaling Up for Mannitol Production by LAB

Good mannitol yields from sugar-rich raw materials have been obtained with yeasts and fungi (Hendriksen et al. 1988; Looten et al. 1992). However, the volumetric productivities are low (2 g/l.h). Another disadvantage of these microorganisms is the prolonged cultivation times needed, which can increase the risk of contamination and the production costs. In contrast, LAB can efficiently produce mannitol from fructose at volumetric mannitol productivities close

to 10 g/l.h, which can be achieved both in batch and in fed-batch cultures (von Weymarn et al. 2003; Racine and Saha 2007). Moreover, Ojamo et al. (2000) achieved volumetric mannitol productivities over 20 g/l.h with LAB by increasing the biomass concentration. However, when mannitol is produced by LAB, about 0.5 mol of additional fructose (or glucose) per mol of fructose consumed for mannitol production is required for NAD(P)<sup>+</sup> regeneration. Thus, the maximum theoretical yield of mannitol from total sugar is 66.7% (von Weymarn et al. 2003). Therefore, von Weymarn et al. (2003) have evaluated the scalability of a new bioprocess for mannitol production by a *Leuc. mesenteroides* strain tested at pilot scale (100l). The production levels achieved were similar to those obtained in preliminary laboratory assays. High-purity mannitol crystals were obtained at similar yield values. The new bioprocess consisting of semi-continuous bioconversion enabled efficient high-yield mannitol production from fructose, obtaining good crystallization yields using simple downstream processing. The production levels achieved in this study were comparable to those of the catalytic hydrogenation processes. The advantages of this bioprocess include low raw material costs, improved mannitol yield from fructose and other sugars, the fact that addition of gases to the reactor is not necessary (hydrogen gas has to be added in the catalytic process), simplified purification protocols, and formation of less by-products per unit of crystalline mannitol produced. Moreover, *Leuc. mesenteroides* is a LAB species commonly used and accepted in the food industry.

#### 11.4.4. Metabolic Engineering for Mannitol, Sorbitol, and Erythritol Production

Biotechnological approaches to increasing sugar alcohol production are summarized in Table 11.1. As described above, mannitol biosynthesis is not a common feature among homofermentative LAB strains. However, Ferain et al. (1996) found that an LDH-negative mutant of *Lact. plantarum*, a LAB species often found in several fermented foods and in the gastrointestinal tract of mammals, produced small amounts of mannitol from glucose unlike its wild-type strain, which was not able to produce mannitol from the same carbohydrate. In addition, high intracellular mannitol production was observed in an LDH-deficient mutant of *L. lactis* (Neves et al. 2000). This strain showed transient accumulation of high amounts of intracellular mannitol (up to 90 mM) and mannitol 1-phosphate (up to 80 mM) in succession, mannitol being metabolized after glucose depletion. The demonstrated ability of producing mannitol by LDH-deficient strains has been used as a tool for increasing mannitol production by metabolically engineered homofermentative LAB strains. To obtain an effective mannitol-producing strain, the mannitol transport system of an LDH-deficient strain was disrupted. This engineering strategy leads to the construction of *L. lactis* strains that are able to produce mannitol in high yields (33%) from glucose (Gaspar et al. 2004). Also, Viana et al. (2005) found that inactivation of the *ldhL* gene in a *Lact. casei* strain produces a misbalance in NADH levels, leading to the activation of other catabolic

**Table 11.1** Overview of biotechnological efforts toward increasing sugar alcohol production.

Sugar alcohol	Species	Type of modification	Reference
Mannitol	<i>Lact. fermentum</i>	Lactate dehydrogenase-deficient mutant	Aarnikunnas et al. (2003)
Mannitol	<i>Lact. plantarum</i>	Lactate dehydrogenase-deficient mutant	Ferain et al. (1996)
Mannitol	<i>L. lactis</i>	Lactate dehydrogenase-deficient mutant and mannitol transport	Gaspar et al. (2004)
Sorbitol	<i>Lact. plantarum</i>	Lactate dehydrogenase-deficient mutant	Ladero et al. (2007)
Mannitol	<i>L. lactis</i>	Lactate dehydrogenase-deficient mutant	Neves et al. (2000)
Sorbitol	<i>Lact. casei</i>	D-sorbitol-6-phosphate dehydrogenase recombinant	Nissen et al. (2005)
Xylitol	<i>L. lactis</i>	Xylose reductase and xylose transporter recombinant	Nyyssölä et al. (2005)
Mannitol	<i>Lact. casei</i>	Lactate dehydrogenase-deficient mutant	Viana et al. (2005)
Mannitol	<i>L. lactis</i>	Overexpression of mannitol-1-phosphate dehydrogenase	Wisselink et al. (2005)

pathways capable of regenerating  $\text{NAD}^+$  without drastically decreasing lactate production. In addition to lactate, the *ldhL* mutant produces pyruvate, acetate, acetoin, ethanol, mannitol, and diacetyl. Thus, in the absence of *ldh*, pyruvate can be channeled mainly through the  $\alpha$ -acetolactate synthase, pyruvate-formate lyase, and pyruvate oxidase pathways. Therefore, pyruvate metabolism has been rerouted, essentially as in other LAB impaired in *ldh*, as described above (Hugenholtz et al. 2002).

Wisselink et al. (2004) have designed a different metabolic engineering approach by overexpressing a mannitol 1-phosphate dehydrogenase gene (*mtlD*) from a *Lact. plantarum* strain into a dairy strain of *L. lactis*. Resting cells of the LDH-deficient transformant were able to convert 25% of glucose into mannitol, which was no longer used after glucose depletion.

Aarnikunnas et al. (2003) constructed different mutant strains (by deleting the D- and L-LDH genes, *ldhD* or *ldhD-ldhL*, respectively) of a strain of *Lact. fermentum* to produce either mannitol and pure L-lactic acid, or pyruvate in a single process. Thus, besides producing mannitol, L-lactate, or pyruvate, valuable substrates for preparing synthetic biopolymers and pharmaceuticals were synthesized. The single mutant produced mannitol and L-lactic acid, showing similar yields and productivities as those of the parent strain. The double mutant strain produced mannitol and pyruvate, but mannitol productivity was lower, although a high mannitol yield was maintained.

Metabolic engineering toward sorbitol production led Nissen et al. (2005) to integrate the D-sorbitol 6-phosphate dehydrogenase-encoding gene (*gutF*) in the lactose operon of a *Lact. casei* strain, following the same regulation of the *lac* genes; resting cells of this recombinant strain are able to synthesize sorbitol from glucose, if they are pre-grown on lactose. The inactivation of the *ldhL* gene increased sorbitol production, suggesting that the engineered route provided an alternative pathway for  $\text{NAD}^+$  regeneration.

The capacity of a *Lact. plantarum* strain to produce sorbitol from fructose 6-phosphate, by reversing the sorbitol catabolic pathway in a mutant

strain deficient for both L- and D-LDH activities, was studied by Ladero et al. (2007). Resting cells under pH control with glucose as substrate showed a rerouting of the flux from fructose 6-phosphate toward sorbitol production, which leads to 65% efficiency of sugar rerouting toward sorbitol biosynthesis; also, mannitol production occurred, although at lower levels (9%–13% glucose conversion), as compared with the control strain. These findings indicate competition for fructose 6-phosphate rerouting by natively expressed M1PDH. Lower levels (25%) of sugar rerouting toward sorbitol were obtained using actively growing cells instead of resting cells. The results of Ladero et al. (2007) indicate that recombinant *Lact. plantarum* strains are promising candidate microorganisms for sorbitol production.

To date, the only evidence of xylitol production by LAB has been achieved by Nyssölä et al. (2005) by expressing the D-xylose reductase from the yeast *Pichia stipitis* CBS 5773, and the xylose transporter from *Lact. brevis* ATCC 8287, in the strain *L. lactis* NZ9800. Xylitol production, which is of great interest in the polyols market, was carried out using high-cell-density cultivation of non-growing recombinant cells under microaerophilic conditions in the presence of xylose and glucose. The xylitol yield from xylose was 1.0 mol/mol, and the ratio of xylitol produced per glucose consumed was 2.5 mol/mol. The volumetric productivity is 2.72 g/l.h after 20 h. Around 34% of the xylose initially present was consumed. A shift from homolactic to mixed-acid fermentation at an early stage of the experiment was observed for the recombinant strain. This new approach shows that LAB could also be promising candidates for efficient xylitol production.

So far, no attempts have been made to increase erythritol production by LAB through metabolic engineering. However, when considering the food-grade nature of these microorganisms, the usefulness of such an approach is evident.

## 11.5. Conclusions

Consumers increasingly demand food products that are not only safe and of high quality but also benefi-



cial for health in one way or another. Functional foods aim to provide this by adding specific attributes that promote intestinal health (probiotics and prebiotics) and reduce the risk of cancer (antioxidants), coronary disease (anti-atherogenic), and diabetes (sugar replacers). Furthermore, an increasing number of people live with debilitating chronic, and usually age-related diseases, such as diabetes, that require specific nutritional attention.

The potential applications of sugar alcohols in the food industry are numerous and vary in nature, not only because of their physicochemical properties but also because of their health-promoting features. Thus, aside from serving as low-calorie sweeteners, sugar alcohols can also act, according to recent claims, as prebiotics.

Many LAB strains have been shown to be ideal cell factories for the production of important nutraceuticals. The development of fermented food products naturally enriched in mannitol through *in situ* production by LAB should be highly beneficial and may lead to novel fermented foods with increased nutritional values. Although many LAB synthesize mannitol naturally, genetic modification strategies help improve its production and, in the case of sorbitol, induce its biosynthesis. Biotechnological processes using microorganisms for these low-calorie sugars are now commercially viable. Undoubtedly, more progress will be made in the production of these low-calorie sugars by LAB and the concomitant introduction of polyol-enriched food products into the market in the near future.

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## Chapter 12

# B-Group Vitamins Production by Probiotic Lactic Acid Bacteria

Jean Guy LeBlanc, María Pía Taranto, Verónica Molina, and Fernando Sesma

*Lactic acid bacteria (LAB), widely used as starter cultures for the fermentation of a large variety of foods, can improve the safety, shelf life, nutritional value, flavor, and overall quality of the fermented products. In this regard, the selection of strains delivering health-promoting compounds (nutraceuticals) is now the main goal of several studies. Among these studies, vitamin production by LAB has recently gained attention of the scientific community. Although most LAB are auxotrophic for several vitamins, it is now known that certain strains have the capability to synthesize B-group vitamins. Humans cannot synthesize most vitamins, and these compounds have to be provided exogenously. Although most essential vitamins are present in a variety of foods, vitamin deficiencies still exist in many countries. Thus, LAB are the ideal candidates for delivering vitamins, among other specific compounds, into foods. Certain fermented milks have shown high levels of B-group vitamins due to LAB biosynthesis. Folate biosynthesis by yogurt starter cultures can increase the “natural” folate levels in this product. These folates do not cause dangerous side effects, such as masking of B<sub>12</sub> deficiencies, as does folic acid, the chemical form of folates commonly used in food fortification. Although little is currently known about riboflavin (vitamin B<sub>2</sub>) production by LAB, the genes involved in their biosynthesis have been identified in several species. Cobalamin (vitamin B<sub>12</sub>), a complex corrin compound, was found to be produced by certain strains of *Lactobacillus reuteri*, particularly CRL1098, a*

*probiotic microorganism. These microorganisms use cobalamin to catabolize glycerol and to produce reuterin, a well-known antimicrobial compound. Since many LAB are recognized as probiotics, their capacity to produce B-group vitamins could be useful for the design of novel functional foods that are able to prevent vitamin deficiencies by enhancing the nutritional value of food products.*

### 12.1. Introduction

Lactic acid bacteria (LAB) are a group of microorganisms that are broadly used as starter cultures for the elaboration of fermented foods. Besides their industrially important fermentative capacities, LAB can also improve the safety, shelf life, nutritional value, flavor, and overall quality of fermented products. In addition, LAB have been shown to exert a large range of beneficial properties, the reason for which they are frequently used as probiotic microorganisms in a variety of novel products. In 2002, the Food Agriculture Organization (FAO) defined probiotics as “live microorganisms which, when administered in adequate amounts, confer a beneficial health effect on the host.” The probiotic and beneficial aspects of LAB have been intensely reviewed elsewhere and will not be the subject of this chapter.

In addition to their intrinsic beneficial properties, certain strains of LAB have the capability of producing, releasing, and/or increasing specific beneficial compounds in foods. These functional ingredients

are sometimes referred to as nutraceuticals, a term that was first coined by Stephen DeFelice in 1989 to describe “a food (or part of a food) that provides medical or health benefits, including the prevention and/or treatment of a disease” (Brower 1998). These ingredients can be macronutrients (such as unsaturated fatty acids present in some oils), micronutrients (such as vitamins), or nonnutritive compounds (such as hydrolytic enzymes and flavonoids) and can be naturally present in foods (such as omega-3 fatty acids in fish or vitamin C in citrus fruits) or added (such as milks fortified with calcium and vitamin D, and cereals fortified with folic acid; Hugenholtz and Smid 2002).

Since LAB are involved in the preparation of a wide range of fermented foods and because of their Generally Recognized as Safe status, the selection of strains delivering health-promoting compounds (nutraceuticals) is now the main objective of several research groups. Among these studies, vitamin production by LAB has recently gained attention of the scientific community. It has been shown that certain foods fermented with LAB contain elevated levels of B-group vitamins as a result of microbial biosynthesis. For this reason, LAB (normally considered food-grade microorganisms) are the ideal candidates for delivering specific compounds such as vitamins into foods.

## 12.2. Vitamins

Vitamins are micronutrients that are essential for the metabolism of all living organisms. They are found as precursors of intracellular coenzymes that are necessary in the regulation of vital biochemical reactions in the cell. Humans cannot synthesize most vitamins, which therefore have to be provided exogenously. Although most vitamins are present in a variety of foods, vitamin deficiencies still exist in many countries including industrialized nations.

Thirteen vitamins are currently recognized as being essential to human health and are classified according to their solubility as either being fat-soluble (vitamins A, D, E, and K) or water-soluble (vitamin C and B-group vitamins). The B-group (or B-complex) vitamins include thiamin, riboflavin,

niacin, pyridoxin, pantothenic acid, biotin, folate, and cobalamin. Each B-group vitamin is chemically different and acts in synergy to maintain the body's homeostasis by playing major roles in metabolic processes such as energy production and red blood cell synthesis. B-group vitamins, normally present in many foods, can easily be removed or destroyed during cooking and food processing, so their deficiencies are omnipresent in many societies. For this reason, many countries have adopted laws requiring the fortification of certain foods with specific vitamins and minerals. For example, in Argentina, the food industry is obligated to fortify all wheat flour for human consumption with iron, folic acid, thiamin, riboflavin, and niacin for the prevention of anemia and neural tube deformation. However, recent reports have shown lack of official controls and that the level of fortification is not always met; as a consequence, serum vitamin levels in the general population have only slightly improved and subclinical deficiencies still persist (ENNyS 2007).

In the following pages, a detailed description of microbial synthesis of three B-group vitamins (riboflavin, folate, and cobalamin) by LAB will be discussed as well their application in food products. Increasing the concentration of vitamins through microbial biosynthesis could be an economically attractive alternative to mandatory fortification of foods.

### 12.2.1. Riboflavin (Vitamin B<sub>2</sub>)

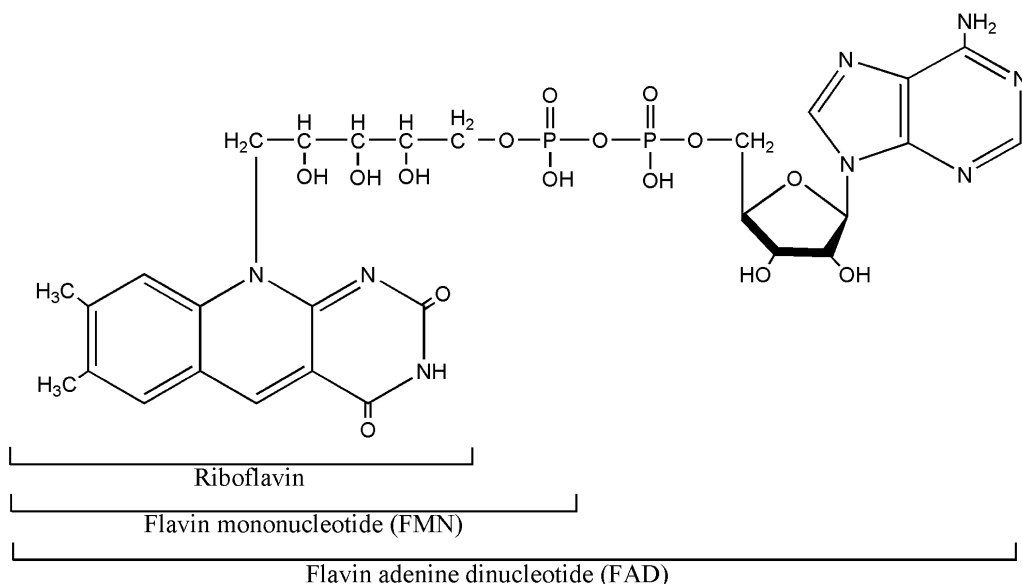
*Chemical structure and requirements.* In 1879, the English chemist Alexander Wynter Blyth published a report on the chemical composition of cow milk, describing for the first time a yellowish pigment that he denominated “lactochrome.” Shortly afterward, similar fluorescent pigments were isolated from a variety of sources (i.e., yeast extracts and eggs), and once their chemical composition was determined, all were found to be derivatives of riboflavin. The name riboflavin derives from the presence of a ribose-like (ribitol) side chain and the word “flavus,” which is Latin for yellow.

Riboflavin (vitamin B<sub>2</sub>) was first identified as being a rat growth-promoting factor. It plays an

essential role in cellular metabolism, being the precursor of the coenzymes flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), which both act as hydrogen carriers in biological redox reactions involving enzymes such as NADH dehydrogenase. The term “riboflavin” will be used to describe all the biologically active forms of vitamin B<sub>2</sub> flavins, including riboflavin, riboflavin-5'-phosphate (FMN), and riboflavin-5'-adenosyldiphosphate (FAD). The riboflavin molecule is formed by an isoalloxazine moiety with a ribitol side chain and is chemically defined as 7,8-dimethyl-10-(1'-d-ribityl) isoalloxazine (Fig. 12.1). This molecule is the basis for all riboflavin derivatives.

The synthesis of the flavin coenzymes from riboflavin is controlled by thyroid hormones that regulate the activities of the flavin biosynthetic enzymes (Rucker et al. 2001). A flavokinase catalyzes the initial phosphorylation from ATP of riboflavin to FMN. FMN can then combine with a second molecule of ATP to form FAD, the predominant tissue flavin, in a reaction catalyzed by FAD synthetase (FAD pyrophosphorylase).

The recommended riboflavin requirements for humans vary depending on sex, age, and physiological state (pregnancy, lactation). Normal adults need to consume between 0.9 and 1.6 mg of riboflavin on a daily basis since the human body cannot adequately store riboflavin (Institute of Medicine 1998). Although riboflavin is present in a wide variety of foods, such as dairy products, meats, eggs, and some green vegetables, riboflavin deficiency (ariboflavinosis) still occurs in both developing and industrialized countries (O'Brien et al. 2001; Blanck et al. 2002). Symptoms of ariboflavinosis in humans include sore throat, hyperemia, edema of oral, and mucous membranes, cheilosis, and glossitis (Wilson 1983). Severe cases of ariboflavinosis are not common in most societies; however, subclinical manifestations are frequent among all subpopulation groups. Subclinical riboflavin deficiencies are only detectable by measuring the vitamin concentration in body fluids such as blood plasma and serum. Vitamin B<sub>2</sub> status in humans has usually been assessed by measuring the erythrocyte glutathione reductase activation coefficient (EGRAC), which is the ratio between glutathione reductase activity



**Figure 12.1** Chemical structures of riboflavin, FMN, and FAD.

**Table 12.1.** Vitamin concentrations in dairy products.

Product	Riboflavin (mg/l)	Folate (μg/l)	Vitamin B <sub>12</sub> (μg/l)
Milk	1.2 ± 0.1	40 ± 10	3.1 ± 0.3
Buttermilk	1.7 ± 0.1	90 ± 20	2.0 ± 0.3
Yogurt	2.0 ± 0.5	80 ± 20	2.0 ± 1.0
Kefir	1.3 ± 0.2	50 ± 10	1.0 ± 0.2
Ropy-milk		110 ± 20	
Sour buttermilk		75 ± 15	
Acidophilus milk		50 ± 10	
Bifidus milk		75 ± 15	

determined with and without the addition of the cofactor, FAD (Glatzle et al. 1970). Glutathione reductase loses FAD at an early stage in vitamin B<sub>2</sub> deficiency, making EGRAC a useful method for the diagnosis of vitamin B<sub>2</sub> deficiency (Bates 1993).

*Riboflavin concentration in fermented foods.* Although dairy products contain riboflavin, they are not considered a good source of this essential vitamin. Considering that milk contains approximately 1.2 mg of riboflavin per liter (Table 12.1), an average adult person and a pregnant woman would need to consume, respectively, 1 and 1.6 liters of milk per day to meet their daily requirement. This level of milk consumption is not normal among residents of industrialized countries such as the United States, where the daily per capita consumption of fresh milk is approximately 200 ml (Putman and Allshouse 2003). In general, increasing the levels of riboflavin in milk would thus be very important to prevent ariboflavinosis in populations where milk consumption is low.

Furthermore, riboflavin concentrations can sometimes vary in certain dairy products due to processing technologies and the action of microorganisms utilized during food processing (Table 12.1). This is the case for buttermilk and yogurt where riboflavin levels increased significantly (1.7 and 2.0 mg/l) compared with unfermented milk (1.2 mg/l). This increase in vitamin B<sub>2</sub> would be due to the action of riboflavin-producing starter cultures during the fermentation process. The proper selection of starter cultures can lead to increased concentration of spe-

cific water-soluble vitamins. It has been shown that certain yogurt starter cultures decreased riboflavin concentrations whereas others could increase the levels of this vitamin to up to 60% of the initial concentration present in unfermented milk (Kneifel et al. 1992). The use of probiotic riboflavin-producing strains would be an interesting alternative for obtaining products enriched with this vitamin in addition to conferring beneficial effects to the host.

*Microbial biosynthesis of riboflavin.* Riboflavin biosynthesis has been described both in Gram (+) and Gram (−) bacteria, with more detail in *Bacillus subtilis* (Perkins and Pero 2002) and *Escherichia coli* (Bacher et al. 1996). The production of riboflavin by some LAB genera, such as *Streptococcus* and *Enterococcus*, isolated from tempeh, has also been described (Keuth and Bisping 1993). Microbial biosynthesis of riboflavin from the precursors guanosine triphosphate (GTP) and D-ribulose 5-phosphate occurs through seven enzymatic steps (reviewed elsewhere; Bacher et al. 2000). The imidazole ring of GTP is hydrolytically opened, yielding a 4,5-diaminopyrimidine, which is converted to 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione by a sequence of deamination, side chain reduction, and dephosphorylation. Condensation of 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione with 3,4-dihydroxy-2-butanone 4-phosphate obtained from ribulose 5-phosphate affords 6,7-dimethyl-8-ribityllumazine. Dismutation of the lumazine derivative yields riboflavin and 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione, which is recycled in the biosynthetic pathway.

Two strategies have been applied to overexpress riboflavin by microorganisms: construction of recombinant strains and use of riboflavin-resistant strains. Perkins et al. (1999) have constructed a series of recombinant *B. subtilis* strains for overproducing riboflavin. A *Corynebacterium ammoniagenes* strain harboring a plasmid containing all of its riboflavin biosynthetic genes accumulating 17-fold as much riboflavin as the host strain has also been constructed (Koizumi et al. 2000). Finally, a B<sub>2</sub>-consuming strain of *Lactococcus lactis* subsp. *cremoris* was converted to a riboflavin “factory” by



**Table 12.2.** Riboflavin produced by microorganisms grown in a chemically defined medium.

Microbial species	Strain	Type <sup>a</sup>	Riboflavin (ug/l)	Reference
<i>Lactococcus lactis</i> subspecies				
<i>L. lactis</i> subsp. <i>cremoris</i>	NZ9000	Native	0	Burgess et al. (2004)
	CB010	Mutant	700	Burgess et al. (2004)
	pNZGBAH	GM	24000	Burgess et al. (2004)
<i>Lactobacillus</i> species				
<i>Lact. plantarum</i>	NCDO1752	Native	0	Burgess et al. (2006)
	CB300	Mutant	600	Burgess et al. (2006)
<i>Propionibacterium</i> species				
<i>P. freudenreichii</i>	B374	Native	200	Burgess et al. (2006)
<i>P. freudenreichii</i>	B2336	Mutant	3000	Burgess et al. (2006)
Other species				
<i>Leuc. mesenteroides</i>	NCDO2028	Native	0	Burgess et al. (2006)
<i>Leuc. mesenteroides</i>	CB207	Mutant	500	Burgess et al. (2006)

<sup>a</sup>GM = genetically modified; Mutant = roseoflavin-resistant mutant.

overexpressing four of its biosynthesis genes (Burgess et al. 2004; Table 12.2).

On the other hand, it has been shown that mutants isolated on the basis of their resistance to the toxic riboflavin analog, roseoflavin, also exhibit a riboflavin-overproduction phenotype (Kukanova et al. 1982). Moreover, roseoflavin has been used to obtain constitutive riboflavin overproducing strains of *B. subtilis* (Perkins and Pero 2002), *L. lactis* (Burgess et al. 2004), *Lactobacillus plantarum*, *Leuconostoc mesenteroides*, and *Propionibacterium freudenreichii* (Burgess et al. 2006; Table 12.2).

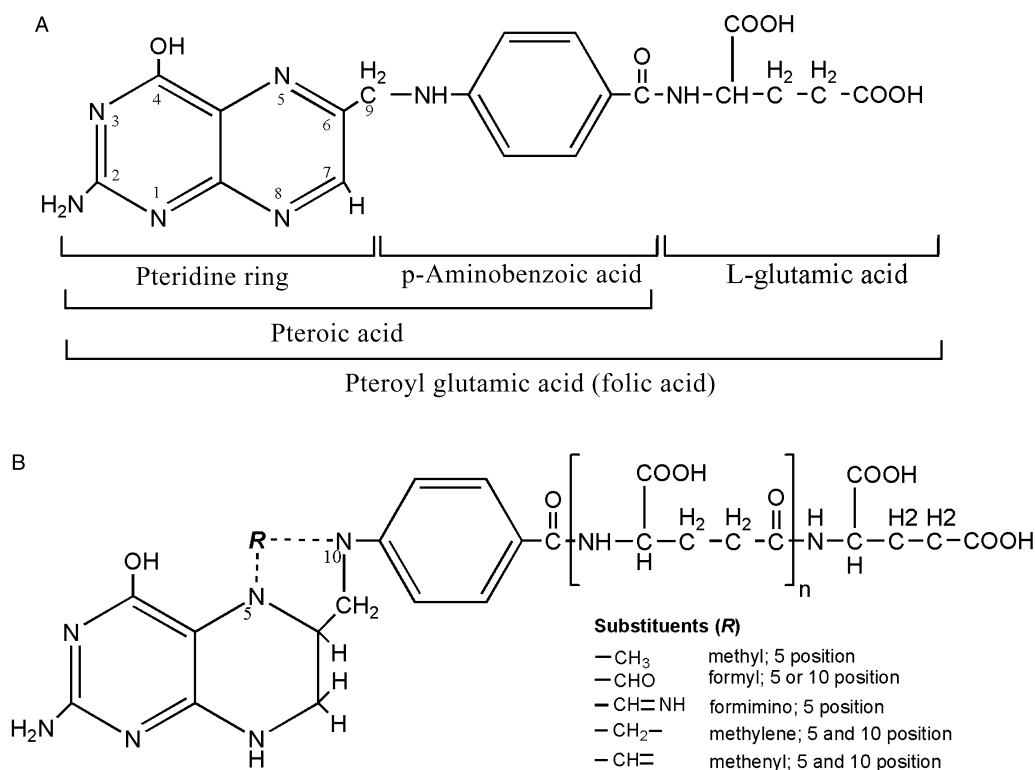
Besides introducing a productive strain, the design of an appropriate fermentation medium is of crucial importance to improving the efficiency and productivity of the riboflavin fermentation process because medium composition can significantly affect product concentration, yield, volumetric production, and the ease and cost of downstream product separation (Kennedy and Krouse 1999).

### 12.2.2. Folates

**Chemical structure and requirements.** Human life cannot exist without folate since this B-group vitamin is involved in essential functions of cell metabolism such as DNA replication; repair and methylation; and synthesis of nucleotides, other

vitamins, and some amino acids. Folates possess antioxidant properties that protect the genome by inhibiting free radical attack of DNA, in addition to their role in DNA repair and replication mechanisms (Duthie et al. 2002). Folate deficiency has been implicated in a wide variety of disorders such as Alzheimer's (Luchsinger et al. 2007) and coronary heart diseases (Danesh and Lewington 1998); osteoporosis (Baines et al. 2007), increased risk of breast (Tjonneland et al. 2006) and colorectal cancers (Duthie et al. 2002), poor cognitive performance (Durga et al. 2007a), hearing loss (Durga et al. 2007b), and neural tube defects (MRC Vitamin Study Research Group 1991; Czeizel and Dudas 1992).

Due to the occurrence of problems associated with current folic acid fortification programs, researchers have been seeking novel methods of increasing concentrations of naturally occurring folates in foods. In this chapter, the generic term folate will include the complete group of all folic acid derivatives, such as the folylglutamates naturally present in foods, and folic acid, which is a synthetic folate form commonly used for food fortification and nutritional supplements. Folic acid or pteroyl glutamic acid is composed of *p*-aminobenzoic acid linked at one end to a pteridine ring and at the other end to L-glutamic acid (Fig. 12.2). The



**Figure 12.2** Chemical structures of (A) folic acid (pteroyl-L-glutamic acid) and (B) native-food folates, for example, reduced, one-carbon-substituted forms of polyglutamates.

naturally occurring forms of folate differ in the extent of the reduction state of the pteroyl group, the nature of the substituents on the pteridine ring, and the number of glutamyl residues attached to the pteroyl group. The naturally occurring folates include 5-methyltetrahydrofolate (5-MTHF), 5-formyltetrahydrofolate (5-formyl-THF), 10-formyltetrahydrofolate (10-formyl-THF), 5,10-methylenetetrahydrofolate (5,10-methylene-THF), 5,10-methyltetrahydrofolate (5,10-methenyl-THF), 5-formiminotetrahydrofolate (5-formimino-THF), 5,6,7,8-tetrahydrofolate (THF), and dihydrofolate (DHF). Most naturally occurring folates are pteroylpolyglutamates, which contain two to seven glutamates joined in amide (peptide) linkages to the  $\gamma$ -carboxyl of glutamate. The principal intracellular folates are pteroylpentaglutamates, while the principal extracellular folates are pteroyl-

monoglutamates. Pteroylpolyglutamates with up to 11 glutamic acid residues exist naturally.

Bioavailability is defined as the proportion of a nutrient ingested that becomes available to the body for metabolic processes or storage. The dietary folate bioavailability may be hampered by the polyglutamate chain to which most of the natural folate is attached (McNulty and Pentieva 2004). This polyglutamate chain must be removed (except for the proximal glutamate moiety) by the enzyme  $\gamma$ -glutamyl hydrolase or human conjugase that is present in the brush border of the small intestine. This enzyme is present in sufficient quantity and is not a limiting factor in folate absorption (Reisenauer and Halsted 1987). Folate can then be absorbed and transported into the portal vein. The existing data suggest that the polyglutamate form is 60%–80% bioavailable compared with the monoglutamate

form (Gregory 1995); it was confirmed in a human trial comparing chemically synthesized heptaglutamyl folate with regular (monoglutamyl) folate (Melse-Boonstra et al. 2004). However, in a rat feeding trial, longer polyglutamyl chain folates (containing an average of eight glutamyl residues) did not show lower, but apparently higher bioavailability, compared with shorter folylpolyglutamates (Sybesma et al. 2003d). This is clearly different from what has been reported for humans (Gregory 1995; McNulty and Pentieva 2004; Melse-Boonstra et al. 2004). One possible explanation is that the rat carboxypeptidase II enzyme that is required for transforming polyglutamyl folates into (monoglutamyl) folates, which can subsequently be absorbed by the blood, does not limit absorption of folates with longer glutamyl tail lengths. Alternatively, the affinity of this enzyme for long polyglutamyl folates might even be higher than for short polyglutamyl folates. Bioavailability can also be affected by folate-binding proteins from milk, which may increase the efficiency of folate absorption by protecting dietary folates from uptake by bacteria in the gut, thus increasing absorption in the small intestine (Eitenmiller and Landen 1999). Other dietary interactions that may affect folate bioavailability include effects of foods on intestinal pH with potential modification of conjugase activity, presence of folate antagonists, intestinal changes influenced by dietary factors, chelation, and factors that influence the rate of gastric emptying. In spite of the large amount of information available on folate bioavailability, knowledge of this important part of folate nutrition has been recently described as fragmentary (Selhub and Rosenberg 1996; Gregory 1997; McNulty and Pentieva 2004).

Humans cannot synthesize folates; in consequence, it is necessary to assimilate this vitamin from exogenous sources. Folates are present in most foods such as legumes (beans, nuts, peas, etc.), leafy greens (spinach), citrus, some fruits, vegetables (broccoli, cauliflower), liver, and (fermented) dairy products (Eitenmiller and Landen 1999). Although beans and green vegetables like spinach are good sources of folates, relatively few people eat enough of these foods. The recommended daily intake (RDI)

of folate in an adult is 200–400 µg (Institute of Medicine 1998; FAO/WHO 2002). For pregnant women, 400–600 µg is recommended. Although folate is omnipresent in a normal human diet, folate deficiencies still occur frequently, even in well-developed countries (Konings et al. 2001; O'Brien et al. 2001). Recent reports have indicated that folate intake levels are inadequate among various population groups including women of childbearing age (Morris and Tangney 2007). Studies such as these have obligated the governing bodies of many countries to advocate mandatory supplementation with folic acid. In Canada and the United States, for example, fortification of flours has been mandatory since 1998 in order to reduce the incidences of neural tube malformations in newborns. Many other countries have afterward developed similar programs. Argentina, for example, has been fortifying its commercial flours with some vitamins, including folic acid since 2002. Even with mandatory folic acid fortification, recent reports have shown that one-third of pregnant and lactating women may not be meeting their folate requirements from diet alone (Sherwood et al. 2006). On the other hand, many countries have not adopted a national folic acid fortification program because of the potential adverse effects of folic acid, particularly that high intakes can delay the diagnosis of vitamin B<sub>12</sub> deficiency (Bailey et al. 2003). The concern is that folic acid is added at levels where individuals with low folate intakes can meet folate RDI in order to prevent neural tube defects and/or lower plasma homocysteine; however, others with normal or higher folate intakes could inadvertently become exposed to excessive folic acid intake, which has the potential to mask the early hematological manifestations of vitamin B<sub>12</sub> deficiency such as pernicious anemia (Sweeney et al. 2007). Other safety considerations of excess folic acid consumption highlighted by the Food and Drug Administration (1996) include potential risks for pregnant women, and persons on anti-epileptic and anti-folate medication. The FDA also noted the uncertainties regarding the effects of chronic elevated exposure in children, whose requirements for folate are lower than those of adults. Further concerns include the potential to

promote cancer (Charles et al. 2004; Kim 2004) and the recent hypothesis that exposure of the fetus to excess folic acid may favor the selection of the methylenetetrahydrofolate polymorphism, which is associated with a range of debilitating illnesses (Luccock and Yates 2005). Natural folates (such as tetrahydrofolates produced by microorganisms) do not cause “masking” of pernicious anemia that occurs at high concentrations of folic acid and should thus be considered a viable alternative to folic acid fortification programs (Scott 1999).

*Folate concentration in fermented foods.* Milk is not a rich source of dietary folate compared with other foods (Table 12.1). However, folate concentrations can be significantly increased in many dairy products processed by microbial fermentations (Table 12.1). In Western countries, consumers are becoming aware that their typical Western diet falls short of the recommended daily allowance of some nutrients. In some population groups, there is a veritable risk of vitamin deficiency, especially among the elderly, since their food intake is lower, and in young children, who consume a restricted variety of foods (Papastoyiannidis et al. 2006). Additionally, increased dietary intakes of folates are suggested, especially for women of childbearing age (Food and Drug Administration 1996). For these reasons, it has been suggested that the fortification of fermented milks with B-complex vitamins seems to be a good option for preventing vitamin deficiencies (Papastoyiannidis et al. 2006). Among dairy products, fermented milks are considered a potential matrix for folate fortification because folate-binding proteins of milk improve folate stability and the bioavailability of both 5-MTHF and folic acid may be enhanced (Jones and Nixon 2002; Aryana 2003; Verwei et al. 2003). However, due to the potential risks of fortification with folic acid, the elaboration of fermented milks containing elevated levels of natural folates would be a better suited alternative.

Besides fermented dairy products, microorganisms are capable of increasing folate content in a wide variety of other foods. For example, fermentation of rye dough to produce bread is often accompanied with an increase in folate content (Kariluoto

et al. 2006). In these studies, the increase in folate content during fermentation was mainly due to folate synthesis by yeasts; LAB did not produce folates but rather consumed them. Variations in starter cultures led to great differences in folate content of sourdoughs, but their effects have been scantily investigated. Proper strain selection, that is, exchanging folate-consuming LAB with folate-producing ones could significantly increase folate content in these breads.

It has also been reported that it is possible to select lactic acid-producing starter cultures that produce significant amounts of 5-MTHF (almost doubling its concentration) during fermentation of vegetables (Jagerstada et al. 2004). To optimize the whole process, it is important to carefully check the folate concentration in the raw vegetables (starting material). In addition, the loss of folate during processing must be limited as much as possible, and the conditions favoring microbiological biosynthesis of folates needs to be better understood and controlled.

*Folate-producing microorganisms.* Numerous studies have shown that industrial starter bacteria (i.e., *L. lactis* and *Streptococcus thermophilus*) have the ability to synthesize folate. They explain why some fermented dairy products, including yogurt, contain higher amounts of folate than nonfermented milk (Table 12.3). However, the ability of commercial yogurt starter cultures to produce or utilize folate has been shown to vary considerably. Although most lactobacilli are unable to synthesize this essential vitamin (Hugenholtz and Kleerebezem 1999; Crittenden et al. 2003), it was demonstrated that *Lact. plantarum* is able to produce folates, although at low levels, when grown in chemically defined folate-free medium (Sybesma et al. 2003c).

The amount of folic acid found in cow milk ranges from 20 to 60 µg/l, whereas its concentration in yogurts may be increased, depending on the starter cultures used and on the storage conditions, to values above 200 µg/l (Wouters et al. 2002). This level depends on the strain of *Strep. thermophilus* and *Lact. delbrueckii* subsp. *bulgaricus* used, because the latter organism has been shown to

**Table 12.3.** Folate produced by microorganisms grown in chemically defined folate-free medium.

Microbial species	Extracellular ( $\mu\text{g/l}$ )	Intracellular ( $\mu\text{g/l}$ )	Total ( $\mu\text{g/l}$ )	Reference
<i>Lactococcus lactis</i> subspecies				
<i>L. lactis</i> subsp. <i>cremoris</i>	8–46	59–99	92–116	Sybesma et al. (2003c)
<i>L. lactis</i> subsp. <i>lactis</i>	5–26	47–269	57–291	Sybesma et al. (2003c)
<i>L. lactis</i> subsp. <i>lactis</i> biovar <i>diacetylactis</i>	14–21	65–84	79–100	Sybesma et al. (2003c)
<i>Lactobacillus</i> species				
<i>Lact. plantarum</i>	27	18	45	Sybesma et al. (2003c)
<i>Lact. helveticus</i>	–1 to 3	–1 to 90	2–89	Sybesma et al. (2003c)
<i>Lact. acidophilus</i>	0	1	1	Sybesma et al. (2003c)
<i>Lact. casei</i>	–45	32	–13	Sybesma et al. (2003c)
<i>Lact. casei</i> subsp. <i>rhamnosus</i>	–98	34	–63	Sybesma et al. (2003c)
<i>Lact. delbrueckii</i> subsp. <i>bulgaricus</i>	12	41	54	Sybesma et al. (2003c)
<i>Propionibacterium</i> species				
<i>P. thoenii</i>	28	8	36	Hugenholtz et al. (2002)
<i>P. acidipropionici</i>	58	–22	36	Hugenholtz et al. (2002)
<i>P. jensenii</i>	51	–11	40	Hugenholtz et al. (2002)
<i>P. freudenreichii</i> ssp. <i>shermanii</i>	0–93	–20 to 41	17–78	Hugenholtz et al. (2002)
<i>P. sp.</i>	13–31	–22 to 16	9–29	Hugenholtz et al. (2002)
<i>Bifidobacterium</i> species				
<i>B. adolescents</i>	1–65	10 to 40	70–110	Pompei et al. (2007a)
<i>B. animalis</i>	26	—	—	Pompei et al. (2007a)
<i>B. bifidum</i>	1	—	—	Pompei et al. (2007a)
<i>B. breve</i>	1–3	—	—	Pompei et al. (2007a)
<i>B. catenulatum</i>	3	—	—	Pompei et al. (2007a)
<i>B. dentium</i>	29	—	—	Pompei et al. (2007a)
<i>B. infantis</i>	27	—	—	Pompei et al. (2007a)
<i>B. longum</i>	2	—	—	Pompei et al. (2007a)
<i>B. pseudocatenulatum</i>	12–82	5–35	75–90	Pompei et al. (2007a)
Other species				
<i>Streptococcus</i> species				
<i>Strep. thermophilus</i>	23–40	4–179	29–202	Sybesma et al. (2003c)
<i>Leuconostoc lactis</i>	37	7	45	Sybesma et al. (2003c)
<i>Leuc. paramesenteroides</i>	33	10	44	Sybesma et al. (2003c)

degrade folates during its growth. It is therefore of utmost importance to select the optimal combination of these strains leading to an organoleptically acceptable yogurt with a concomitantly increased folic acid concentration. It is now known that not only yogurt starter cultures and *L. lactis* have the ability to produce folates, but only this important property exists in other LAB. *Lact. acidophilus* is reported as being able to increase folates in fermented milks (Lin and Young 2000). As mentioned before, *Lact. plantarum* is capable of producing folates in chemically defined medium (Table 12.3), and thus it should be evaluated if this LAB can

increase folate levels in milk. Additionally, recent reports have shown that some probiotic microorganisms, such as bifidobacteria, have the ability to synthesize folates (Lin and Young 2000; Crittenden et al. 2003; Holasova et al. 2004; Pompei et al. 2007a) and propionibacteria (Hugenholtz et al. 2002; Holasova et al. 2004; Table 12.3). The oral administration of folate-producing probiotic strains may confer a more efficient protection against inflammation and cancer, both by exerting the beneficial effects of folate and by delivering it to colonic-rectal cells (Pompei et al. 2007a). In humans, folate produced by the microbiota in the small intestine is



assimilated by the host (Camilo et al. 1996). Although folate is believed to supply only a minor source of total absorbed folate in humans (Bates 1993), the contribution of the microbiota to the folate requirements of the high cell turnover intestinal epithelium is unknown. A mechanism for luminal folate absorption by cells in the human colon has been reported (Dudeja et al. 1997), which suggests that folate produced *in situ* by the colonic microbiota may be utilized by cells in the colonic epithelium. Asrar and O'Connor (2005) showed that bacterially synthesized folate are absorbed across the large intestine and incorporated into the liver and kidneys of piglets. These authors predicted that approximately 18% of the dietary folate requirement for the piglet could be met by folate absorption across the large intestine. Also, increased intestinal *Bifidobacterium* populations, induced by consumption of human breast milk, have been correlated with an enhanced folate status in rats (Krause et al. 1996). It is therefore possible that probiotic bacteria active in the intestinal tract may be able to contribute to the folate requirement of colonic epithelial cells. However, further research is required to determine if these bacteria produce folate in the intestinal environment; the form in which this folate occurs; the availability of this folate for transport and utilization by colonocytes from the lumen; and the contribution of the intestinal microbiota to the total folate requirement of colonic epithelial cells.

The genes for folate biosynthesis have been identified in *L. lactis* (Sybesma et al. 2003b), *Lact. plantarum* (Kleerebezem et al. 2003), and *Lact. delbrueckii* subsp. *bulgaricus* (van de Guchte et al. 2006). The biosynthetic pathway in LAB includes seven consecutive steps, to convert the precursor GTP into tetrahydrofolate (Suzuki and Brown 1974). However, in some LAB that cannot synthesize folate, some of the genes involved in folate biosynthesis are lacking in the genome; this is the case for *Lact. gasseri* (Wegkamp et al. 2004), *Lact. salivarius* (Claesson et al. 2006), *Lact. acidophilus*, and *Lact. johnsonii* (van de Guchte et al. 2006).

It has been shown that metabolic engineering can be used to increase folate levels in *L. lactis* (Sybesma et al. 2003b; Wegkamp et al. 2007), *Lact. gasseri*

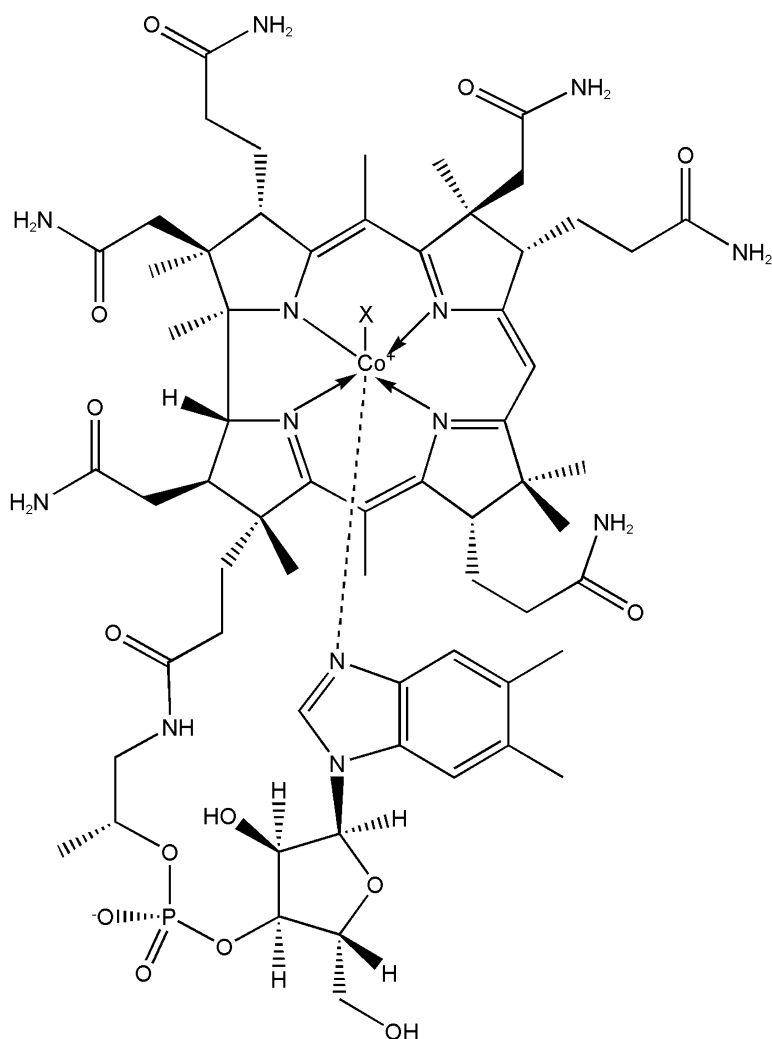
(Wegkamp et al. 2004), and *Lact. reuteri* (H. Santos et al., pers. comm.).

### 12.2.3. Vitamin B<sub>12</sub> (Cobalamin)

The term vitamin B<sub>12</sub> (B<sub>12</sub>) is generally used to describe a type of cobalt corrinoid, particularly of the cobalamin group. In strict terms, vitamin B<sub>12</sub> is the form of the vitamin that is obtained during industrial production and that does not exist naturally (Rucker et al. 2001). Cyanide stabilizes the molecule during the extraction procedure from microbial cultures, forming cyanocobalamin. In its natural form, the vitamin is present principally as desoxyadenosilcobalamin (coenzyme B<sub>12</sub>), methylcobalamin, and pseudocobalamin, among other forms. Structurally, the cobalamin molecule can be divided in three parts (Fig. 12.3): the central corrinic ring with the four ligands of a cobalt ion; a superior (or beta) ligand that is attached to the adenosyl o methyl group, and the lower ligand (or alpha), normally dimethylbenzimidazole (DMB); however, in several anaerobic bacteria, adenine and other ligands can also be present, forming pseudocobalamin (or pseudo-B<sub>12</sub>) and other active cofactors (Martens et al. 2002).

Although vitamin B<sub>12</sub> was described for the first time by Minot and Murphy (2001) and isolated by Rickes et al. (1948), it was Hodgkin et al. (1956) who revealed the magnificent structure of the most complex of the vitamins.

Animals, plants, and fungi are incapable of producing cobalamin; it is the only vitamin that is exclusively produced by microorganisms, particularly by anaerobes (Roth et al. 1996; Martens et al. 2002; Smith et al. 2007). Furthermore, biochemical and genomic data indicate that only a few bacteria and archaea possess the ability to produce this vitamin (Roth et al. 1996; Rodionov et al. 2003). Adult ruminant animals and strict vegetarians can obtain the vitamin from specialized bacteria present in the rumen. Humans, however, do not have such microflora in their small intestine and must absorb the coenzyme from natural sources such as animal meats (especially liver and kidney), fish, eggs, and pharmaceutical products (Herbert 1996).



**Figure 12.3** Chemical structure of vitamin B<sub>12</sub>.

Vitamin B<sub>12</sub> deficiency can cause different pathological manifestations that affect the hematopoietic, neurological, and cardiovascular systems, among others. One of the most extreme forms of B<sub>12</sub> deficiency is known as pernicious anemia, which is not normally associated to diet but rather to problems in the gastric system caused by a lack of production of a gastric glycoprotein called intrinsic factor, which facilitates the absorption of the vitamin in the small intestine (Beck 2001).

*General characteristics of vitamin B<sub>12</sub>.* Vitamin B<sub>12</sub>, in all of its different forms, such as adenosylcobalamin (AdoCbl) or methylcobalamin, is used as a cofactor in three principal types of catalytic reactions (Kräutler 2005): (1) intramolecular rearrangements (mutations), for example, glutamate mutase, methylmalonil CoA mutase, ornitin mutase, L-lisine mutase, and methylglutarate mutase; (2) reduction of ribonucleotides to deoxyribonucleotides, for example, ribonucleotide reductase; and (3)

methyations, for example, methionine synthetase that catalyzes the synthesis of methionine.

In humans and other superior animals, B<sub>12</sub> acts as a cofactor in only two reactions: methionine synthase and methylmalonyl CoA mutase. According to their vitamin B<sub>12</sub> requirements, bacteria have been separated in three distinct groups (Roth et al. 1996): (1) those that require cobalamin to grow and that can synthesize it *de novo* (*Pseudomonas denitrificans*); (2) those that require cobalamin to grow but are unable to synthesize it (*Lact. delbrueckii* subsp. *lactis*); and (3) those that cannot synthesize vitamin B<sub>12</sub> nor need it (*L. lactis*).

With the constant apparition of new microbial genomic sequences being published, it can be inferred that several microorganisms do not produce B<sub>12</sub> but possess (using *in silico* evaluations) the ability to transport and utilize B<sub>12</sub> (Rodionov et al. 2003), although it is not always clear in which reaction this vitamin is involved.

**Biosynthesis of cobalamin.** As indicated above, only bacteria and archaea are able to synthesize B<sub>12</sub>, although relatively few can synthesize it *de novo*. One of the first model organisms used for the study of B<sub>12</sub> biosynthesis was *P. freudenreichii*, which is used in the industrial production of the vitamin. However, the first experiments with this bacterium were not fruitful due to the instability of the biosynthesis intermediates (Battersby 1994). In order to circumvent this problem, an important step was the use of the aerobic B<sub>12</sub>-producing bacteria *Ps. denitrificans*, which allowed the isolation of numerous intermediates and the characterization of the majority of the genes involved in the biosynthesis of this vitamin (Battersby 1994; Thibaut et al. 1998). These studies concluded that the biosynthesis of cobalamin could be performed in either (a) aerobic or oxygen-dependent or (b) anaerobic or oxygen-independent conditions.

The anaerobic route, observed in strains of *P. freudenreichii*, *Salmonella enterica*, and *Bacillus megaterium*, was recently described (Warren et al. 2002; Warren 2006; Escalante-Semerena 2007). The initial characterization problems were due principally to the fact that the central Co ion was inserted

into the corrinoid ring in an early step that generated unstable intermediates that were difficult to isolate. On the other hand, in the oxygen-dependent route, cobalt is inserted in a later stage, creating more stable intermediates.

The biosynthesis of cobalamin can be divided into three steps. The first involves the synthesis of uroporphyrinogen III, which is a common intermediate in the synthesis of various tetrapyrrolic cofactors such as heme, chlorophyll F, and siroheme. The second consists of the synthesis of the corrinic ring. Two variations exist in this step according to its aerobic or anaerobic synthesis. As mentioned above, in this step the cobalt is quelated. The third step is common for both the anaerobic and aerobic routes, where the corrinic ring is adenosylated, an aminopropanol group is attached, and the nucleotidic loop is assembled that links on the bottom of the corrinic ring. The lower ligand is generally DMB in humans, in animals, and in bacteria such as *Ps. denitrificans*, whereas pseudo-B<sub>12</sub> and other cofactors can be found as a cofactor in microorganisms that use the oxygen-independent route of synthesis.

Due to the complexity of B<sub>12</sub> biosynthesis and to the limitations of the scope of this chapter, interested readers are invited to read excellent reviews that have been published on this subject (Raux et al. 2000; Scott 2003; Roessner and Scott 2006; Escalante-Semerena 2007).

**Cobalamin biosynthesis by *Lactobacillus reuteri*.** It is well known that certain strains of *Lact. reuteri* are capable of metabolizing glycerol in co-fermentation with glucose. The first enzyme in this metabolic route is glycerol dehydratase, which requires AdoCbl as a cofactor (Daniel et al. 1998). In this reaction, glycerol is converted to 3-hydroxypropionaldehyde (3-HPA), which is subsequently reduced by NADH to 1,3-propanediol (1,3-PDL) mediated by the 1,3-PDL:NAD oxidoreductase. It was shown that *Lact. reuteri* CRL1098 was able to metabolize glycerol in a B<sub>12</sub>-free medium, this being the first indirect evidence of the presence of intracellular cobalamin in *Lact. reuteri* (Taranto et al. 2003). The chromatographic analysis of the intracellular bacterial extract of *Lact. reuteri* CRL1098 confirmed that

this strain was able to produce a compound with an absorption spectrum closely identical to standard cobalamin but with a different elution time. The presence of cobalamin or of one of its intermediates in the intracellular extract of *Lact. reuteri* CRL1098 was also confirmed using different bioassays (Taranto et al. 2003).

Genetic evidence of cobalamin biosynthesis by *Lact. reuteri* CRL1098 was then obtained through the use of different molecular biology techniques. The genetic organization (*cob* and *chi* genes) are very similar to those of *S. enterica* and *Listeria innocua* (Santos et al. 2007). It is important to note that the denomination of the genes for the biosynthesis of cobalamin has been confused since some researchers have used different prefixes, *chi* and *cob*, to describe the genes in *Ps. denitrificans* and *S. enterica*, respectively. Since some of the genes are present in both biosynthetic routes, these can have different denominations depending on which route is being described.

The genetic representation of the biosynthesis cluster of *Lact. reuteri* CRL1098 is shown in Figure 12.4 (see in the color plate section), where at least 30 genes are involved in the *de novo* synthesis of the vitamin. A distinctive characteristic of the *cob* cluster of *Lact. reuteri* is the presence of *hem* genes in the middle of the cluster. In the respiratory organisms *Listeria* and *Salmonella* with similar *cob* clusters, the *hem* genes are described on other regions of their genome. The presence of the *hem* genes in the *cob* cluster is a characteristic that was observed only in certain genomes of *Clostridium* (Rodionov et al. 2003).

Additionally, transcriptional analysis performed in *Lact. reuteri* CRL1098 showed that the highest levels of expression of the *cob* cluster was observed in the late exponential growth phase in the absence of cobalamin, although the existence of exogenous cobalamin only partially repressed the expression of the operon. Recently, the transcription of a vast set of genes involved in cobalamin synthesis in sourdough prepared with strain *Lact. reuteri* ATCC 55730 was described (Hufner et al. 2008).

In addition to the strain CRL1098, other *Lact. reuteri* strains were also shown to be capable of

producing some corrinoids such as *L. reuteri* DCM 20016 (Santos et al. 2008), JCM1112 (Morita et al. 2008), and CRL1324 and CRL1327 strains isolated from vagina (Vannini et al. 2008).

*Types and amount of B<sub>12</sub> produced by Lact. reuteri* CRL1098. Bioassays have been used to confirm that *Lact. reuteri* produces cobalamin in the order of 50 µg/l, which is a modest amount compared with the values obtained by the strains used in industrial production that surpass 200 mg/l (Martens et al. 2002). However, these values could be considered adequate when the daily requirement of vitamin B<sub>12</sub> is taken into account (2–3 µg for an adult) because they could be sufficient for the development of foods bio-enriched with B<sub>12</sub>. A possible drawback in the technological development is that the major form of the corrinoid produced in anaerobiosis by *Lact. reuteri* is pseudocobalamin (Santos et al. 2007) which, although present as a coenzyme in many anaerobic and facultative anaerobic bacteria, is not active in animals and humans (Brandt et al. 1979; Rucker et al. 2001). Recent data indicate that in microaerophilic conditions *Lact. reuteri* CRL1098 not only produces pseudo-B<sub>12</sub>, but also produces a small amount of other corrinoids including coenzyme B<sub>12</sub> (Vera 2007).

*Glycerol and 1,2 propanediol fermentation: evidence of a cob-pdu genomic island.* As described above, *Lact. reuteri* is able to co-metabolize glycerol and glucose anaerobically to produce 1,3-propanediol (Talarico et al. 1990). One intermediate product of the first reaction in the metabolic route mediated by the B<sub>12</sub>-dependent glycerol dehydratase is an antimicrobial compound denominated reuterin, which is an equilibrium mixture of different monomeric and dimeric forms of 3-HPA (Talarico et al. 1988; Talarico and Dobrogosz 1989). The use of glycerol does not represent an energy gain for the cell. At least in *Lact. reuteri* CRL1098 the biomass of the culture grown in glycerol-glucose is lower than when grown in glucose alone. Therefore, it is possible that the metabolism of this polyol and the production of reuterin are associated with an ecological advantage for the bacterium that would allow

it to compete in complex ecosystems such as the gut. This is supported by another study (Morita et al. 2008), who demonstrated that *Lact. reuteri* JCM 1112 (DSM20016) is able to produce reuterin *in vivo* in the gut of gnotobiotic mice under physiological conditions.

In addition to glycerol, *Lact. reuteri* can also use 1,2-propanediol in the presence of other fermentable sugars, giving rise to final products such as propanol and propionate (Sriramulu et al. 2008). In that work it was confirmed that glycerol (or diol) dehydratase was associated with mini-compartments called metabolosomes, whose structural proteins are also encoded by genes found inside the *pdu* operon (propanediol utilization). As for glycerol, 1,2-propanediol can be found as a substrate in human and animal intestines and principally come from the metabolism of sugars derived from plants and mammalian glycoconjugates, such as rhamnose and fucose (Bobik et al. 1999; Bobik 2006). Also, it was shown that 1,2 propanediol was able to partially support the growth of *Lact. reuteri* DSM20016 in de Man, Rogosa and Sharpe basal medium without glucose (Sriramulu et al. 2008).

This information indicates that both substrates, present in the intestines, could be important for *Lact. reuteri*, to release an antimicrobial substance and as an alternative substrate that can partially be catabolized for bacterial growth. Furthermore, the *cob-pdu* cluster would be part of a genomic island that was acquired by horizontal transfer in some strains of *Lact. reuteri* (Morita et al. 2008; Santos et al. 2008). This acquisition would confer a competitive advantage compared with microorganisms that do not possess this cluster. The horizontal transfer hypothesis of the *cob-pdu* cluster was previously suggested for some strains of enterobacteria (Roth et al. 1996).

***B<sub>12</sub> production by other LAB.*** After the detection of the production of B<sub>12</sub> by *Lact. reuteri* CRL1098 and the study of its cobalamin biosynthesis cluster, the genomic sequence of two strains of *Lact. reuteri*, with different characteristics, were released by the Joint Genome Institute: *Lact. reuteri* F275 (type strain DSM20016) isolated from human feces that is unable to colonize mouse, and *Lact. reuteri* 100-23

isolated from mouse intestines. Curiously, comparative genomic data revealed that the strain isolated from human intestines (DSM 20016) presented the cobalamin biosynthesis cluster that was also associated with the anaerobic catabolism of glycerol (or 1,2-propanediol), whereas the mouse strain (100-23) did not contain the *cob* nor the glycerol metabolism genes.

Based on the horizontal transfer hypothesis of the *cob-pdu* cluster, it would be expected that other strains of LAB would have also received this genomic island by one of the many mechanisms of genetic transfer. Again, from the current sequenced genomes of LAB, only *Lact. reuteri* contain the *pdu-cob* genes (DSM20016/JCM1112 and the Biogaia strain *Lact. reuteri* ATCC55730). Recently, Martin et al. (2005) showed that a reuterin-producing strain of *Lactococcus coryniformis* isolated from goat milk was able to produce a cobalamin-type compound. Preliminary genetic and biochemical data from our laboratory (Vannini et al. 2008) indicate that the *cob-pdu* cluster would be spread in other lactobacilli (*Lact. coryniformis*, *Lactococcus murinus*, and *Lactococcus curvatus*). The possibility of holding various vitamin B<sub>12</sub>-producing strains and species of LAB would be important, not only for future basic studies on cobalamin production, but also evolutionary studies related with the acquisition of the *cob-pdu* genomic island, and its potential application in the development of products that contain B<sub>12</sub>.

According to the Integrated Microbial Genomes System, more than 250 microbial genomes (including more than 20 LAB genomes) have been sequenced to date, with several other projects ongoing and more in the process of being launched (Markowitz et al. 2006). With information from these sequencing projects, such as those published by the Joint Genome Institute Microbial Sequencing program ([http://genome.jgi-psf.org/mic\\_home.html](http://genome.jgi-psf.org/mic_home.html)), it is now possible to analyze specific bacterial genomes *in silico* in order to determine if they contain the complete metabolic pathways necessary for *de novo* vitamin biosynthesis. A number of useful tools can be found on the Internet that are of great help in analyzing microbial genomes. One



of the more useful sites is that of the Genome Analysis and System Modeling Group of the Life Sciences Division of Oak Ridge National Laboratory (<http://genome.ornl.gov/microbial/>). This site contains tools that allow organization of genes in function of metabolic pathways, functional categories, taxonomic distribution, and so on. In the metabolism of cofactors and vitamins subcategory, it is possible to see which enzymes are present in the genome compared with the reference metabolic pathways, thus permitting the user to determine if a specific microorganism has the ability to biosynthesize riboflavins, folates, and vitamin B<sub>12</sub>, without performing laboratory assays. Similar tools are available at the Kyoto Encyclopedia of Genes and Genomes databases (<http://www.genome.jp/kegg/>).

### 12.3. Vitamin Bioavailability Studies: Animal Models

#### 12.3.1. Riboflavin

Riboflavin-deficient rat models have been extensively used to study the biological effects of riboflavin. Using these models, it has been shown that riboflavin (1) is important in the early postnatal development of the brain (Ogunleye and Odotuga 1989) and gastrointestinal tract (Williams et al. 1996; Yates et al. 2003); (2) is able to modulate carcinogen-induced DNA damage (Pangrekar et al. 1993; Webster et al. 1996); (3) plays a role in iron absorption and use (Butler and Topham 1993; Powers et al. 1993); and (4) can modulate inflammatory responses (Lakshmi et al. 1991). These models also allow the extrapolation of data to human clinical data (Greene et al. 1990). Because riboflavin is essential in various biochemical reactions that are related to obtaining metabolic energy from carbohydrates and fatty acids, the deficiency of this vitamin can cause severe symptoms in animals such as stunted growth (Glatzle et al. 1970).

By using similar animal models, it has been demonstrated that riboflavin produced by LAB can effectively revert the biological effects of an induced deficiency of this vitamin. In one study, riboflavin-producing strains (native and genetically modified) of *L. lactis* were able to eliminate most physiologi-

cal manifestations of ariboflavinosis such as stunted growth, elevated EGRAC values, and hepatomegalia, which were observed using a riboflavin depletion-repletion model (LeBlanc et al. 2005a). Similarly, riboflavin status and growth rates were greatly improved when the depleted rats were fed cultures of *L. lactis* that overproduced this vitamin, whereas the control strain did not show the same effect (LeBlanc et al. 2005b).

Other studies have been performed to evaluate the physiological response of feeding with fermented milks elaborated with riboflavin-producing strains. The administration of a fermented milk that was produced with a spontaneous riboflavin overproducing strain of *P. freudenreichii* (strain B2336) was able to improve animal growth compared with conventional yogurt and with the same product fermented with the parental wild-type strain, which produces low levels of this essential vitamin (LeBlanc et al. 2006). The fermented product containing *P. freudenreichii* B2336, with increased levels of riboflavin, eliminated most physiologic manifestations of ariboflavinosis observed when using a riboflavin depletion-repletion model (i.e., stunted growth, high EGRAC values, and hepatomegalia). The product fermented with the non-riboflavin-producing strain did not show the beneficial effect (LeBlanc et al. 2006).

Also, the administration of milk fermented by *L. lactis* pNZGBAH, a genetically engineered strain that overproduces riboflavin, showed the same reversion against ariboflavinosis (LeBlanc et al. 2005b).

#### 12.3.2. Folate

Different experimental animal models have been used to evaluate the biological effects of folate-producing microorganisms. In one study, folate-overproducing bifidobacteria (*Bifidobacterium adolescentis* MB 227, *B. adolescentis* MB 239, and *B. pseudocatenulatum* MB 116), administered to Wistar rats with induced folate deficiency, exerted both the beneficial effects of probiotics and *in vivo* folate production, positively affecting the folate status of rats (Pompei et al. 2007b).

In other studies, the bioavailability of different folates produced by engineered *L. lactis* strains was evaluated using a rodent depletion-repletion bioassay. Rats were fed a folate-deficient diet and different *L. lactis* cultures were added as a source of folate (Sybesma et al. 2003a). The folate produced by the engineered *L. lactis* was able to compensate for the folate depletion in the diet and showed similar bioavailability as externally added (chemical) folic acid. Folate levels in some organs and blood (liver, kidneys, and serum) increased significantly in animals that received the folate-producing strains compared with those that did not receive bacterial supplementation. In this rat bioassay, biological folates present in the food bacterium *L. lactis* showed similar or even better bioavailability than the chemically synthesized folic acid normally used for food fortification. This demonstrates that fermented dairy products would represent an excellent source of folates in the diet.

*L. lactis* strains have also been modified to produce intracellularly folates with a short glutamyl tail length (average polyglutamyl tail length of 3) or with a long polyglutamyl tail length (average polyglutamyl tail length of 8), which generates an increased retention of folate in the cells (Sybesma et al. 2003d). These strains were evaluated in animal models in order to determine if the folate glutamyl tail length affects bioavailability. As stated before, in contrast to (monoglutamyl) folate, polyglutamyl folates cannot be transported across the cell membrane. Hence, the release of intracellular polyglutamyl folate depends on the disruption of the cells during passage through the gastrointestinal tract. The clear responses to lactococcal cells added to a folate-free diet of deficient rodents on the folate levels in organs and blood indicate that these cells lyse after consumption and that the bacterial folate becomes available for absorption in the gastrointestinal tract of the rat (Sybesma et al. 2003a). This study provided the first animal trial with food-containing living bacteria that were engineered in order to increase the intracellular accumulation of folate or to change the average polyglutamyl tail length compared to a wild-type lactococcal strain. This study revealed that *L. lactis* could be used to deliver

and release folates in the gastrointestinal tract, a result also obtained previously with riboflavin using *L. lactis* (LeBlanc et al. 2005a) and *P. freudenreichii* (LeBlanc et al. 2006).

### 12.3.3. Vitamin B<sub>12</sub>

Recently, a vitamin B<sub>12</sub>-deficient murine experimental model was developed to evaluate maternal B<sub>12</sub> deficiency from the end of the gestation period to weaning (Molina et al. 2008). In this experimental animal model, vitamin B<sub>12</sub> deficiency caused a significant reduction in the hematological parameters (hemoglobin, hematocrit, and reticulocytes values) and anthropometric alterations in pregnant females compared with the control animals, which were fed a B<sub>12</sub>-sufficient diet. Moreover, the deficient females gave birth to smaller numbers of offspring, which also showed growth retardation (smaller size) and a decrease in hematological values. The vitamin B<sub>12</sub> deficiency caused histological alterations in the small intestine and a decrease in the number of IgA-producing cells of the females and in their offspring. This experimental murine model constituted by females and their offspring allowed the assessment of the incidence of maternal cobalamin deficiency in offspring and would be a useful tool for evaluating the efficiency of functional foods containing B<sub>12</sub>-producing microorganisms to prevent the nutritional deficit of cobalamin.

As indicated above, *Lact. reuteri* CRL1098 produces a compound with B<sub>12</sub> activity (Taranto et al. 2003). High performance liquid chromatography coupled with a diode array detector, mass spectrometry and nuclear magnetic resonance spectroscopy allowed the identification of the main component as Co $\alpha$ -[ $\alpha$ -(7-adenyl)]-Co $\beta$ -cyanocobamide or pseudovitamin B<sub>12</sub> (Santos et al. 2007). Although pseudovitamin B<sub>12</sub> is the corrinoid produced in anaerobic condition, a small amount of B<sub>12</sub> was observed in microaerobic conditions (Vera 2007). This effect was also observed in *S. enterica* and other microorganisms. This is due to the oxygen-dependent synthesis of 5,6-dimethylbenzimidazole, the lower ligand moiety of cobalamin (Renz 1999).

In order to determine if the vitamin produced by strain CRL1098 is bioavailable, an animal model was implemented to evaluate the efficiency of the compound(s) produced by this strain to prevent the symptoms caused by a nutritional cobalamin deficient diet in pregnant female mice and their weaned offspring (Molina et al. 2009). The females that received the deficient diet plus LAB improved their general condition with weight gains and hematological values similar to those of the normal group females (control). The prevention of the B<sub>12</sub> deficiency in the pregnant females fed the probiotic strain together with the deficient diet was evidenced particularly in the offspring. These weaned young showed normal hematological values and anthropometric parameters (weight and size) similar to the young from normal females. The serum vitamin B<sub>12</sub> level was similar to the values found in the normal young. It is important to emphasize that the number of offspring was the same as in the case of the normal females. Moreover, the intestinal damage and the minor number of IgA+ cells produced by the vitamin deficiency were prevented both in the pregnant females and in their respective offspring by the consumption of the producer strain together with the B<sub>12</sub>-deficient diet. These results constituted strong evidence of the way in which the nutritional status of the mother affects the normal development of her offspring, and demonstrated clearly that the vitamin produced by *Lact. reuteri* CRL1098 is biologically active (Molina et al. 2009). In addition, a successful implementation of this model was applied in a food system, using a novel soymilk beverage containing the B<sub>12</sub>-producer strain. Soybean does not contain B<sub>12</sub>; therefore, the fermented soy-product prepared with the B<sub>12</sub>-producing *Lact. reuteri* is a good model for evaluating the bioavailability of the vitamin produced by this microorganism using the above mentioned experimental murine model (Molina et al. 2009). Results confirmed that mice that were fed this fermented product containing *Lact. reuteri* CRL1098 were able to prevent the different pathologies associated with the vitamin deficiency in the same manner as mice fed a soy-product without *Lact. reuteri* but supplemented with commercial B<sub>12</sub>.

## 12.4. Conclusions

This chapter describes that increased levels of B-group vitamins in yogurts and fermented milks are possible through judicious selection of the microbial species and cultivation conditions. It is expected that the food industry will take the next step to use this information for selecting vitamin-producing strains as part of their starter cultures to produce fermented products with elevated levels of these essential compounds. Such products would provide economic benefits to food manufacturers since increased “natural” vitamin concentrations would be an important value-added effect without increasing production costs. Consumers would obviously benefit from such products since they could increase their vitamin intake while consuming them as part of their normal lifestyle. The proper selection of probiotic vitamin-producing strains provides a strategy for the development of novel functional foods with increased nutritional value.

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## Chapter 13

# Bioactive Peptides Derived from Casein and Whey Proteins

Elvira María Hebert, Lucila Saavedra, and Pasquale Ferranti

*Recently, milk proteins have attracted more attention in the biological and medical research community not only because of their nutrient value but also as substances that constantly supply the organism with bioactive compounds. Bioactive peptides are hidden in a latent state within the primary sequences of casein and whey proteins, requiring enzymatic proteolysis for their release. Bioactive peptides can be produced during in vivo gastrointestinal digestion or food processing. Therefore, peptides with physiological effects are largely found in fermented milks and cheeses. The proteolytic system of lactic acid bacteria used as starter cultures in the manufacture of yogurt, fermented milks, and cheeses contributes to the release of bioactive peptides or their precursors. Antimicrobial, immunomodulatory, antithrombotic, opioid, and antioxidant activities; enhancement of mineral absorption and/or bioavailability; and blood pressure-lowering effect are some of the biological activities attributed to milk-derived peptides. Certain milk-derived peptides are commercially produced and used as dietary supplements in functional foods, personal care products, or drugs. The great complexity and the wide range of peptide abundance in these products severely challenge the capabilities of existing analytical methodologies. A major step forward in this field has been achieved by combining the biochemical and biological approach with the tools of proteomics technology. In this review, the most recent achievements will be presented, leading to a deeper*

*definition of the structure-activity relationship in these complex peptide systems.*

### 13.1. Introduction

Recently, extensive scientific evidence (Hayes et al. 2007a) has been provided for the existence of biologically active peptides and proteins derived from foods that might have beneficial effects on human health. In this sense, food proteins have attracted more attention in the biological and medical research community not only because of their nutrient value but also as substances that constantly supply the organism with bioactive compounds (Kitts and Weiler 2003).

Casein is the main protein component of milk, constituting about 80% (2.7 g/100 g milk) of the total bovine milk protein fraction. Although neither casein nor individual casein fractions have any established physiological role, peptides derived from casein have been shown to possess different biological activities. Antimicrobial, immunomodulatory, antihypertensive, opioid, and mineral-binding are some of the biological activities ascertained for milk derived-peptides (Meisel 2004).

### 13.2. Generation of Bioactive Peptides

Biologically active or functional peptides are food-derived peptides that exert, beyond their nutritional value, a physiological, hormone-like effect in the

body. Milk protein-derived bioactive peptides are inactive within the sequence of the primary protein and can be released and activated by enzymatic proteolysis *in vivo* or *in vitro*. There are several ways that encrypted peptides can be released from milk proteins: (1) *in vivo* by enzymatic hydrolysis with digestive enzymes such as trypsin and chymotrypsin; (2) *in vivo* during digestion by microbial enzymes; (3) *in vitro* by endogenous enzymes present in milk; (4) *in vitro* by proteolysis with enzymes derived from microorganisms or plants; and (5) *in vitro* during food processing or ripening by proteolytic starter cultures or enzymes isolated from proteolytic microorganisms (e.g., *Lactobacillus helveticus* and *Lactobacillus delbrueckii* subsp. *lactis*) (Yamamoto et al. 1999; Hebert et al. 2008). A combination of enzymatic hydrolysis by gastrointestinal digestion and fermentation of milk with proteolytic starter cultures or proteolysis using enzymes derived from microorganisms or plants has been demonstrated to be effective in producing short functional peptides (Korhonen and Pihlanto 2007).

### 13.2.1. Production of Bioactive Peptides during Milk Fermentation

The most important application of lactic acid bacteria (LAB) is their use as starter cultures in the manufacturing processes of various fermented dairy products. In addition, certain LAB, mainly the strains belonging to the genera *Lactobacillus*, are increasingly marketed as health-promoting, that is, probiotic bacteria (Saxelin et al. 2005), while certain *Lactobacillus* strains can release bioactive health-beneficial peptides from milk proteins (Meisel 2004; Hayes et al. 2007a). LAB have a limited capacity to synthesize amino acids and are therefore dependent on the use of exogenous nitrogen sources for optimal growth (Hebert et al. 2000, 2004). As milk contains only small amounts of amino acids and short peptides, LAB depend on a complex proteolytic system to obtain essential amino acids from caseins during growth in milk. This specialized proteolytic system consists of a cell envelope-associated proteinase (CEP), specialized transport systems to allow uptake of the resulting peptides, and several intracellular

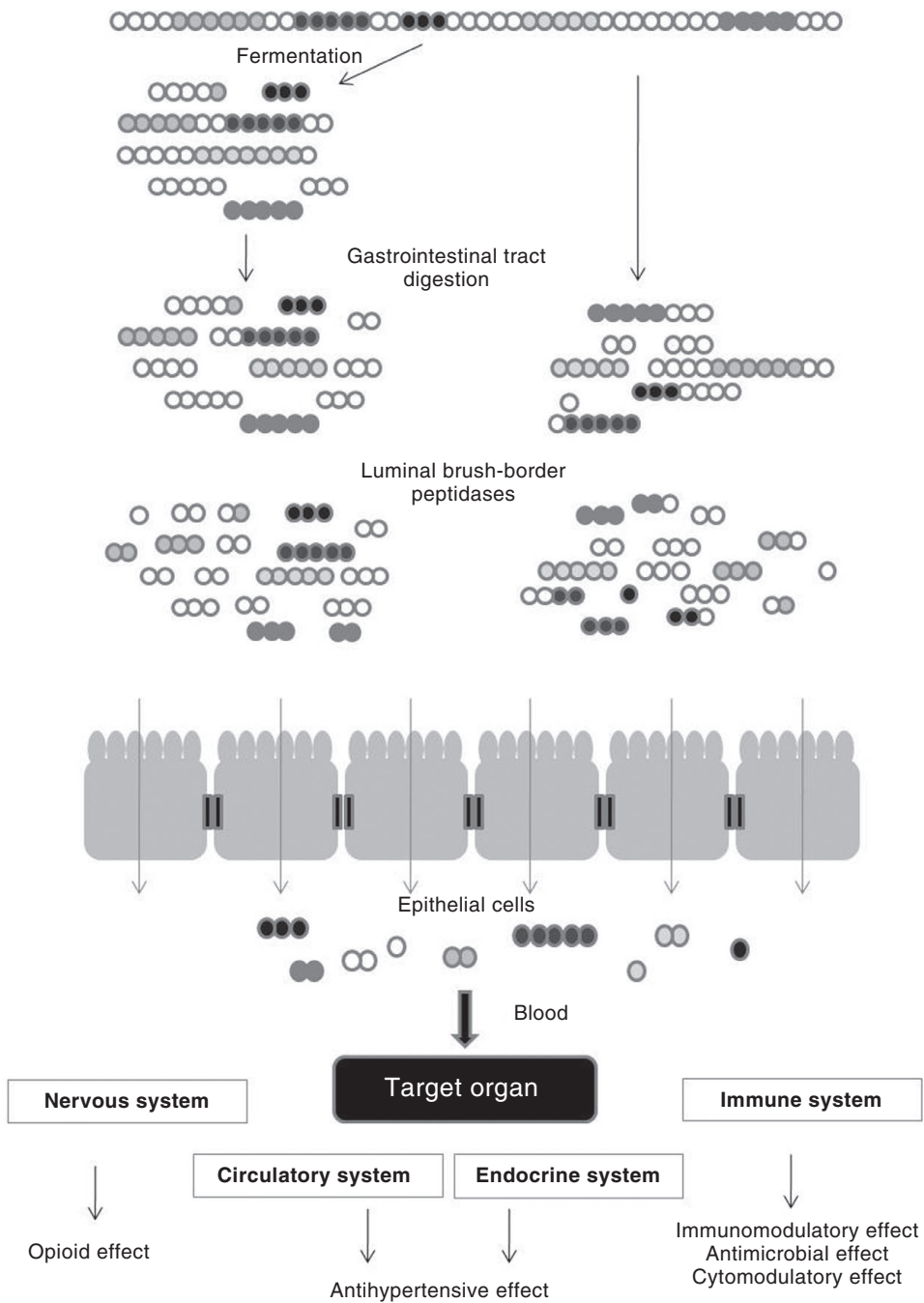
peptidases, which degrade peptides to amino acids (Kunji et al. 1998; Savijoki et al. 2006). In milk fermentation processes, the CEP is the key enzyme of this system as it is involved in the first step of casein degradation and enables LAB to grow in milk, thereby ensuring successful fermentation. In addition to its vital role for bacterial growth in milk, certain CEPs can release bioactive health-beneficial peptides during milk fermentation (Hata et al. 1996; Hayes et al. 2007a; Hebert et al. 2008).

### 13.2.2. Fate of Bioactive Peptides

Ingested proteins are first hydrolyzed in the acid environment of the stomach, where low pH initiates protein hydrolysis and gastric pepsin cleaves proteins into large peptides (Fig. 13.1). The next stage of luminal protein digestion comprises the small intestinal digestion by the major enzymatic secretions from the pancreas trypsin, chymotrypsin, elastase, and carboxi-peptidases. The luminal digestion of proteins results in free amino acids and 3 to 6 amino acid peptides, which could have biological activity. Subsequent brush-border peptidases at the surface of the epithelial cells digest peptides into dipeptides and tripeptides and free amino acids. Brush-border peptidases include enterokinase, aminopeptidases, and dipeptidases. However, certain peptides are resistant to the action of proteolytic enzymes and remain intact in the intestinal tract, producing local effects (Shimizu 2004). Therefore, following digestion, bioactive peptides can either produce local effects in the gastrointestinal tract or be absorbed through the intestine to enter intact into the blood circulation and exert systemic effects.

Different transport systems for the absorption of peptides have been described. Oligopeptides seem to be transported by endocytosis, although it has been reported that more than 90% of the transported peptides are hydrolyzed in the absorptive cells (Heyman and Desjeux 1992). In addition, oligopeptides could be passively transported via paracellular pathways, which is known to be the main mechanism for transport of intact peptides (Pappenheimer et al. 1994). Dipeptides and tri-peptides are actively transported via a specific transport system that exists





**Figure 13.1** Fate of peptides released from precursor proteins by fermentation and/or gastrointestinal digestion.

in the brush-border membrane, PepT1, (Leibach and Ganapathy 1996). Whichever the mechanism used, a variety of peptides would reach the portal circulation, exerting diverse physiological functions (Fig. 13.1).

### 13.3. Biological Activities of Milk-derived Peptides

Depending on the sequence of amino acids, the bioactive peptides can exhibit diverse activities, which have been described *in vitro* and *in vivo*. Moreover, several ingredients and dairy functional foods with function claims based on bioactive peptides are commercialized (Table 13.1).

#### 13.3.1. Antihypertensive Peptides

Hypertension, or high blood pressure, is one of the major risk factors for cardiovascular disease, such

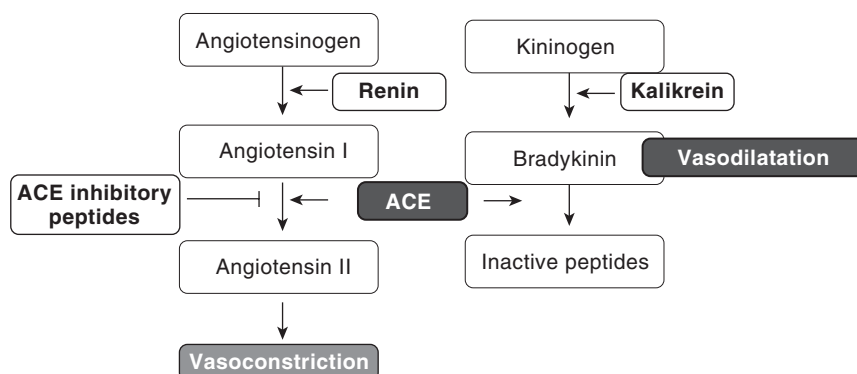
as myocardial infarction, stroke, and heart failure (Erdmann et al. 2008). In recent studies, blood pressure variability has been shown to be a more important determinant of target organ damage than high blood pressure itself (Tatasciore et al. 2007). Clinical trials have shown that in hypertensive subjects, lowering the blood pressure reduces the risk of cardiovascular diseases (Collins et al. 1990; FitzGerald et al. 2004).

Angiotensin I-converting enzyme (ACE) plays a crucial role in the regulation of blood pressure and acts mainly via the renin–angiotensin system, with angiotensin I and bradykinin as the natural substrates (Fig. 13.2). ACE (peptidyl dipeptide hydrolase, EC 3.4.15.1), an exopeptidase that cleaves dipeptides from the C-terminal side of various oligopeptides, increases blood pressure by converting the decapeptide angiotensin I into the potent

**Table 13.1.** Examples of commercially available dairy functional foods with function claims based on bioactive peptides.

Brand name	Manufacturer	Type of product	Claimed bioactive peptides	Observed bioactivity
Calpis	Calpis Co., Tokyo, Japan	Sour milk	IPP, VPP	ACE inhibitory activity, antihypertensive
Evolus	Valio, Helsinki, Finland	Calcium-enriched fermented milk	IPP, VPP	ACE inhibitory activity, antihypertensive
Casein DP	Kanebo Ltd., Tokyo, Japan	Casein hydrolysate	FFVAPFPEVFGK	Antihypertensive properties
C12 peptide	DMV, Veghel, the Netherlands	Casein hydrolysate/ingredient	FFVAPFPEVFGK	Antihypertensive properties
BioZate	Davisco, Eden Prairie, MN	Whey protein hydrolysate	Whey-derived peptides	Antihypertensive properties
BioPURE-GMP	Davisco	Whey protein isolate	Glycomacropeptide ( $\kappa$ -casein f106-109)	Anticariogenic, antimicrobial, antithrombotic
Lactium/PRODIET F200	Ingredia, Arras, France	Capsule, tablets, powders, chewing-gums, drinks	YLGYLEQLLR <sup>a</sup>	Anti-anxiety, anti-stress properties
CE90 GMM	DMV	Ingredient	CPP (19%)	Helps mineral absorption
Capolac	Arla Foods Ingredients, Viby, Denmark	Ingredient	CPP	Helps mineral absorption
Recaldent (CPP-ACP)	Cadbury, England	Ingredient/gums	CPP	Anticariogenic, helps remineralize the enamel
Vivinal Alpha	Friesland Foods Domo, Zwolle, the Netherlands	Ingredient	Whey-derived peptides	Aids relaxation and sleep

<sup>a</sup>Information gathered from Hartmann and Meisel (2007).  
IPP, Ile–Pro–Pro; VPP, Val–Pro–Pro.



**Figure 13.2.** The renin–angiotensin system.

vasoconstricting octapeptide angiotensin II, which induces release of aldosterone and therefore increases sodium concentration and furthers the blood pressure. In addition, ACE degrades bradykinin, which has vasodilatory properties. By inhibiting these processes, synthetic ACE inhibitors have long been used as antihypertensive agents. Since many drugs have side effects, research has been devoted toward producing foods with ACE inhibitory peptides, which are beneficial for individuals who have high blood pressure (FitzGerald et al. 2004). In recent years, some food proteins have been identified as sources of ACE inhibitory peptides, milk products being the main source of these bioactive peptides (FitzGerald et al. 2004). These are currently the best known class of bioactive peptides and have received considerable attention for their effectiveness in both the prevention and the treatment of hypertension (Hong et al. 2008).

ACE inhibitors are thought to be competitive substrates for ACE. Although there is no apparent consensus on the peptide sequence for the expression of ACE inhibitory activity, some common structural properties of ACE-inhibitory peptides are worth noting (Cheung et al. 1980). The ACE binding is strongly influenced by the C-terminal tripeptide sequence of the substrate, and it seems that ACE prefers substrates or competitive inhibitors containing hydrophobic amino acids including aromatic amino acids such as Trp, Tyr, and Phe, or the amino acid Pro located at the three C-terminal positions. In

addition, the positive charge from Arg and/or Lys residues may increase the inhibitory activity (Pripp et al. 2004). In general, ACE inhibitory peptides are short peptides with only two to nine amino acids and most are dipeptides or tripeptides, which are resistant to the action of digestive-tract endopeptidases (Kitts and Weiler 2003). In milk proteins in particular, low-molecular-weight peptides containing Pro residues exhibit very strong ACE inhibitory activity (Saito 2008). The sequence Tyr–Pro is frequently present at the C-terminal region of bovine caseins ( $\alpha_{s1}$ -casein f146–147 and f159–160;  $\beta$ -casein f114–115; and  $\kappa$ -casein f58–59). ACE inhibitory peptides from caseins and whey proteins are termed casokinins and lactokinins, respectively.

Numerous studies in spontaneously hypertensive rats (SHR) as well as in hypertensive human volunteers have been performed to demonstrate the antihypertensive effect of milk-derived ACE inhibitors. The casokinin tripeptides Ile–Pro–Pro and Val–Pro–Pro, produced during milk fermentation by *Lact. helveticus* CP790 or LBK, reduced blood pressure in SHR after a single (Nakamura et al. 1995) or long-term oral administration (Sipola et al. 2002), while milk fermented with *Lact. helveticus* CP791, a variant defective for proteinase activity, did not affect the systolic blood pressure of SHR. Fermented milk produced by *Lact. helveticus* CHCC637 and *Lact. helveticus* CHCC641 reduced mean arterial pressure by about 12 mmHg more than the control product in SHR (Fuglsang et al. 2002). Tyr–Pro

dipeptide, purified from a *Lact. helveticus* CPN4 fermented yogurt-like product, also produced acute antihypertensive effects in SHR after a single oral dose when systolic blood pressure was measured by the tail-cuff method (Yamamoto et al. 1999). Interestingly, this fermented product contains neither Ile-Pro-Pro nor Val-Pro-Pro, which are found in sour milk fermented over a longer period.

Five peptides that were isolated from a proteinase K digestion of whey protein exhibited antihypertensive effects in SHR after gastric intubation (Abubakar et al. 1998). Their amino acid sequences and sources are the following: Val-Tyr-Pro-Phe-Pro-Gly ( $\beta$ -casein; f59-64), Gly-Lys-Pro ( $\beta$ 2-microglobulin; f18-20), Ile-Pro-Ala ( $\beta$ -lactoglobulin [BLG]; f78-80), Phe-Pro (serum albumin; f221-222;  $\beta$ -casein, f62-63, f157-158, and f205-206), and Val-Tyr-Pro ( $\beta$ -casein; f59-61). Chemical synthesis of these peptides confirmed their antihypertensive activity in SHR. The synthetic tripeptide Ile-Pro-Ala, originating from  $\beta$ -lactoglobulin (f78-80), showed the strongest antihypertensive activity. In addition, the tetrapeptide Ala-Leu-Pro-Met ( $\beta$ -lactosin B), derived from the BLG f142-145, showed an antihypertensive activity in SHR after oral administration (Murakami et al. 2004).

In clinical trials, it was shown that *Lact. helveticus*-fermented milks containing Ile-Pro-Pro and Val-Pro-Pro tripeptides have reduced systolic and diastolic blood pressure in hypertensive subjects (Hata et al. 1996; Seppo et al. 2003; Mizushima et al. 2004; Jauhiainen et al. 2007). Similarly, tablets containing Ile-Pro-Pro and Val-Pro-Pro tripeptides have also been shown to have a tendency to lower blood pressure (Aihara et al. 2005; Mizuno et al. 2005).

The presence of ACE inhibitors peptides has also been reported in several cheeses including Parmigiano-Reggiano (Addeo et al. 1994), Gouda (Saito et al. 2000), Manchego (Gomez-Ruiz et al. 2004), Qula, a traditional Tibetan acid curd cheese (Jiang et al. 2007), and the low-fat cheese "Festivo" (Ryhänen et al. 2001). Although some studies showed the production of ACE inhibitory peptides during cheese ripening, extended ripening periods could result in the degradation of ACE inhibitory

peptides (Ong and Shah 2008). Recently, Butikofer et al. (2008) have determined the contents of the antihypertensive peptides Val-Pro-Pro and Ile-Pro-Pro in 101 samples from 10 different Swiss cheese varieties using high-performance liquid chromatography (HPLC) with subsequent triple quadrupole mass spectrometry (MS) analysis. These traditional cheese varieties of Swiss origin such as Gruyère, Sbrinz, Emmental, Appenzeller, and Vacherin fribourgeois contain, on average, similar concentrations of the two antihypertensive peptides to the recently developed fermented milk products with blood pressure-lowering property (Butikofer et al. 2008). In addition, the tripeptide Ile-Pro-Pro has been identified in the  $\beta$ -casein hydrolysate generated by the CEP of *Lact. delbrueckii* subsp. *lactis* CRL 581 (Hebert et al. 2008). On the other hand, the casein hydrolysate containing both antihypertensive peptides, Val-Pro-Pro and Ile-Pro-Pro, improved the vascular endothelial dysfunction, independent of blood pressure, in subjects with mild hypertension (Hirota et al. 2007).

The potency of an ACE inhibitor is usually expressed as an  $IC_{50}$  value, which is the inhibitor concentration leading to 50% inhibition of ACE activity. The majority of milk protein-derived ACE inhibitors have moderate inhibitory potencies, usually within an  $IC_{50}$  range of 100–500  $\mu$ mol/l (Hong et al. 2008). To exert an antihypertensive effect, the bioavailability of ACE inhibitors is of vital importance. Most of the peptides in the enzymatic hydrolysate of food proteins are degraded by host peptidases during digestion and absorption; as a consequence, some peptides could lose their biological activity by the action of gastrointestinal, brush-border, serum, and blood proteinases and peptidases before reaching the blood in an active form. Contrariwise, some peptides, which are not active before ingestion, can be converted to their active form by limited proteolysis. Hence, peptide activities, which are detected using *in vitro* assays, cannot directly express a variety of beneficial activities observed with *in vivo* trials. Another important observation from the *in vivo* trials is that the consumption of specific hydrolysates or fermented dairy product peptides has little or no effect on

blood pressure of normotensive subjects, suggesting that the ACE inhibitory milk-derived peptides exert no acute hypotensive effect (FitzGerald et al. 2004). Consequently, ACE inhibitory peptides could be applied as initial treatment in mildly hypertensive individuals or as supplemental treatment. In addition, these antihypertensive peptides have lower ACE inhibitory activity *in vitro* than the ACE inhibitory drugs, and do not have the harmful side effects reported for synthetic ACE inhibitors, such as dry cough, skin rashes, and angioedema (Hong et al. 2008). ACE inhibitory peptides represent a low-cost alternative treatment for hypertension and, consequently, lower health-care costs.

### 13.3.2. Antithrombotic Peptides

Another complication related to cardiovascular diseases is the tendency to develop thrombosis due to abnormalities in coagulation. Thrombosis can manifest as sudden death, acute coronary syndromes, secondary effects to coronary atherosclerosis, post-operative deep vein thrombosis, and activated protein C deficiency, among other disorders (Gurm and Bhatt 2005). Antithrombotic drugs are commonly used to reduce platelet aggregation and to enhance fibrinolysis.

Amino acid sequence similarities exist between the fibrinogen  $\lambda$ -chain and  $\kappa$ -casein, and there are also functional similarities between milk and blood coagulation (Jolles 1975; Jolles et al. 1986). Blood and milk clotting processes are similar, both involving limited proteolysis; in blood, thrombin cleaves fibrinogen to produce fibrin and fibrinopeptides, whereas in milk, chymosin cleaves  $\kappa$ -casein to form para- $\kappa$ -casein and glycomacropeptide (GMP). To date, food-derived peptides with antithrombotic properties are mainly the result of enzymatic hydrolysis of bovine  $\kappa$ -casein (Jolles et al. 1986).

The main antithrombotic peptide Met-Ala-Ile-Pro-Pro-Lys-Lys-Asn-Gln-Asp-Lys, isolated from the bovine  $\kappa$ -casein, corresponds to the residues 106 to 116 and is termed casoplatelin (Jolles et al. 1986). Casoplatelin inhibits both aggregation of ADP-treated platelets and binding of fibrinogen to ADP-treated platelets; its behavior is similar to

that of the structurally related C-terminal dodecapeptide of human fibrinogen  $\gamma$ -chain (Jolles et al. 1986). Three amino acid residues (Ile<sub>108</sub>, Lys<sub>112</sub>, and Asp<sub>115</sub>) of the casoplatelin seem to be important for the antithrombotic effect because they are homologous in positions to the  $\gamma$ -chain sequence of human fibrinogen (Fiat and Jolles 1989). The two smaller tryptic peptides ( $\kappa$ -casein f106–112 and f113–116) exerted a minimal effect on platelet aggregation and did not inhibit fibrinogen binding. Several  $\kappa$ -casein-derived glycopeptides from different origins, including human and sheep, have also been shown to inhibit platelet aggregation (Qian et al. 1995a, 1995b). The C-terminal part (residues 106–171) of sheep  $\kappa$ -casein inhibits thrombin- and collagen-induced platelet aggregation in a dose-dependent manner. Three peptides, Lys-Asp-Gln-Asp-Lys (residues 112–116), Thr-Ala-Gln-Val-Thr-Ser-Thr-Glu-Val (residues 163–171), and Gln-Val-Thr-Ser-Thr-Glu-Val (residues 165–171), obtained from an enzymatic hydrolysate of GMP, completely inhibited thrombin-induced platelet aggregation (Qian et al. 1995b). Moreover, a peptide derived from a pepsin digest of sheep and human lactoferrin was shown to inhibit thrombin-induced platelet aggregation (Qian et al. 1995a). A tetrapeptide, Lys-Arg-Asp-Ser, from human lactotransferrin, which has structural similarities to fibrinogen  $\alpha$ -chain, has been shown to inhibit *in vitro* platelet aggregation but to a lesser extent than the fibrinogen analogue, Arg-Gly-Asp-Ser (Mazoyer et al. 1990). Lys-Arg-Asp-Ser is an inhibitor of serotonin release by a mechanism independent of protein phosphorylation and an inhibitor of fibrinogen binding (Mazoyer et al. 1990). Therefore, Lys-Arg-Asp-Ser and Arg-Gly-Asp-Ser have different mechanisms of action, and/or their binding sites are different and sequence-specific. In *in vivo* studies, Lys-Arg-Asp-Ser was an inhibitor of arterial thrombus formation in dogs (Wu et al. 1992). In rats, Lys-Arg-Asp-Ser was more effective than Arg-Gly-Asp-Ser in inhibiting thrombus formation at low concentrations, whereas in guinea pigs the reverse was the case (Drouet et al. 1990).

Milk protein-derived antithrombotic peptides would be absorbed intact into the bloodstream.



Bovine and human  $\kappa$ -caseinoglycomacropeptides have been identified in physiologically active concentrations in the plasma of newborn infants after ingestion of cow-milk-based formula or human milk, respectively (Chabance et al. 1995). In adult humans, the GMP was also detected in plasma after milk or yogurt ingestion (Chabance et al. 1998).

### 13.3.3. Immunomodulatory Peptides

There has been increased interest in the study of the relationship between nutrition and immunity due to the hypothesis that consumption of specific foods may reduce susceptibility to the establishment and/or progression of immunological diseases. Many immunomodulatory peptides are derived from the main milk proteins, the caseins. In an early study, it was shown that a hexapeptide obtained from human casein by trypsin-digestion, with the structure Val-Glu-Pro-Ile-Pro-Tyr, stimulated *in vitro* the phagocytosis of opsonized sheep red blood cells by murine peritoneal macrophages (Parker et al. 1984). Furthermore, this hexapeptide, when administered intravenously, enhanced the resistance of mice to *Klebsiella pneumoniae* infection (Parker et al. 1984).

Bovine milk is known to contain a number of immunoregulatory peptides that have been characterized, and they correspond mainly to the residues 106–109 of  $\kappa$ -casein (caseinomacropeptides); residues 194–199 of  $\alpha_{s1}$ -casein; and residues 63–68, 191–193, and 193–202 of  $\beta$ -casein. Both suppressive and enhancing effects on immune variables have been found (Parker et al. 1984; Migliore-Samour and Jolles 1988). In an earlier study, it was shown that the mitogenic activity of a polypeptidic fraction obtained by pepsin-chymosin treatment of bovine caseins, on primed lymph node cells and unprimed spleen cells of rats, was located in residues 193–209 of  $\beta$ -casein (Coste et al. 1992). Sandré et al. (2001) demonstrated that this peptide upregulated a major histocompatibility complex class II antigen expression on bone marrow-derived macrophages, increases their phagocytic activity, and induced only a small release of cytokines. From

these outcomes, Sandré et al. suggested that the peptide  $\beta$ -casein (193–209) might exert an anti-infectious immunostimulating activity without proinflammatory effects through a modulation of macrophage properties. This peptide has been identified in the  $\beta$ -casein hydrolysate generated by the CEP of *Lactococcus lactis* (Juillard et al. 1995). The *in vitro* stimulation of macrophages required relatively high doses of peptide, but due to the high quantity of casein in cow milk, such levels could be obtained during milk fermentation. In addition, the immunomodulatory peptides,  $\beta$ -casein f193-209 and f192-209, were isolated from yogurt, fermented milks, and different types of cheeses such as Feta and Camembert (Dionysius et al. 2000).

Casein, hydrolysed by *Lactobacillus* GG and digestive enzymes (pepsin and trypsin), has been reported to yield compounds possessing both stimulating and suppressing effects on lymphocyte proliferation (Sutas et al. 1996). Whereas  $\alpha_{s1}$ - and  $\beta$ -caseins suppressed the proliferation of lymphocytes,  $\kappa$ -casein stimulated its proliferation. On the other hand, a milk permeate medium containing  $\beta$ -casein as the only source of protein and fermented by a highly proteolytic strain of *Lact. helveticus* was able to modulate the *in vitro* proliferation of lymphocytes by acting on the production of cytokines (Laffineur et al. 1996). LeBlanc et al. (2002) demonstrated the immuno-enhancing and antitumor properties of peptides released during fermentation of milk with the proteolytic strain *Lact. helveticus* R389. The humoral immune response in mice was assessed by measuring the number of IgA-secreting cells, whereas the antitumor response was monitored by studying the regression of subcutaneously implanted fibrosarcomas. The administration of three peptide fractions, isolated from the *Lact. helveticus* R389 fermented milk, increased significantly the IgA-producing cell count and inhibited the growth of fibrosarcoma, as was shown by a decrease in tumor volume compared with normally fed mice (LeBlanc et al. 2002). Additionally, the protective humoral immune response after an *Escherichia coli* O157:H7 infection in mice was also demonstrated for these peptidic fractions. *Lactobacillus paracasei* NCC2461 was found to induce oral tolerance to BLG

*in vivo* by degrading acidic peptides and releasing immunomodulatory peptides (less than 100kDa), stimulating regulatory T cells, which function as major immunosuppressive agents by secreting IL-10. *Lact. paracasei* peptidases have been shown to hydrolyze tryptic–chymotryptic peptides from BLG, releasing numerous small peptides with immunomodulating properties (Prioult et al. 2004).

#### 13.3.4. Opioid Peptides

Food peptides with opioid activity have been classified as exorphins due to their exogenous origin and morphine-like activity. The opioid peptides have been divided in two groups: “typical” and “atypical” opioid peptides. Typical endogenous opioid peptides or endorphins are originated from proopi-melanocortin, proenkephalin (yielding enkephalin), and prodynorphin (yielding endorphin and dynorphin). Structurally, typical opioid peptides have the same N-terminal amino acid sequence, Tyr-Gly-Gly-Phe, which is the fragment that is able to interact with opioid receptors. Atypical opioid peptides have an N-terminal Tyr residue, but the rest of the N-terminal amino acid sequence is not identical to that of the typical opioid peptides (Teschemacher 2003). Opioid peptides have affinities to bind to opiate receptors. At least three types of these receptors have been described:  $\mu$ - (morphine),  $\delta$ - (enkephalin), and  $\kappa$ - (dynorphin) receptors. Opioid receptors have been found in the central and in the peripheral nervous systems, in the immune system, and in the endocrine system of mammals (Teschemacher 2003). The  $\mu$ -type receptor is responsible for emotional behavior and peristalsis affecting intestinal transport of electrolytes; the  $\kappa$ -receptor is also thought to be responsible for emotional behavior, while the  $\delta$ -receptor is thought to be responsible for sedation, analgesia, and food intake (Hayes et al. 2007b).

The caseins and whey proteins are potential sources of opioid peptides. Milk-derived peptides, generated by hydrolysis of various precursor proteins, are atypical opioid peptides. If these peptides resist the hydrolysis by intestinal brush-border

enzymes, they can exert direct effects on specific gastrointestinal target receptors. Alternatively, they can be absorbed intact into the blood circulation and can produce an effect after reaching endogenous opioid receptors. The major opioid peptides are fragments of  $\beta$ -casein, called  $\beta$ -casomorphins, derived from fragments of the  $\beta$ -casein sequence 60–70, which corresponds to the amino acid sequence Tyr-Pro-Phe-Pro-Gly-Pro-Ile-Pro-Asn-Ser-Leu.  $\beta$ -casomorphins have been characterized mainly as  $\mu$ -type ligands (Teschemacher 2003) and have been shown to cause analgesia, apnea, and changes in the sleeping patterns of neonatal rats (Meisel and FitzGerald 2000).  $\beta$ -Casomorphins were found in analogous positions in sheep, water buffalo, and human  $\beta$ -casein (Meisel and FitzGerald 2000). An analogue isolated from a commercial hydrolysate of bovine casein,  $\beta$ -casomorphin-4-amide, designated as morphiceptin, has been shown to provide spinal analgesia and a decrease in arterial blood pressure and heart rate, in addition to depressing motor behavior (Paroli 1988). Three  $\alpha$ -casein-derived exorphins corresponding to bovine  $\alpha_{s1}$ -casein peptide fragments 90–96 (Arg-Tyr-Leu-Gly-Tyr-Leu-Glu), 90–95, and 91–96 are  $\delta$ -selective receptor ligands. Chiba et al. (1989) identified a bioactive peptide, called casoxin C, with opioid antagonist activity from  $\kappa$ -casein (residues 35–41). Opioid peptides have also been obtained from bovine whey proteins (Chiba and Yoshikawa 1986), and they behave as  $\mu$ -opioid receptor agonist with low potency. These peptides are  $\alpha$ -lactorphin (Tyr-Gly-Leu-Phe) and  $\beta$ -lactorphin (Tyr-Leu-Leu-Phe) corresponding to residues 50–53 in both bovine and human  $\alpha$ -lactalbumin, and 102–105 in bovine BLG, respectively (Chiba and Yoshikawa 1986; Antila et al. 1991).

Several studies have shown that certain LAB are capable of releasing opioid peptides during milk fermentation. The extracellular  $P_1$ -type proteinase of *Lact. lactis* hydrolyzed  $\beta$ -casein into more than 100 different oligopeptides generating the  $\beta$ -casein oligopeptide f60–68, which forms part of  $\beta$ -casomorphin-11 (Juillard et al. 1995). An opioid peptide,  $\beta$ -casomorphin 1–4 (f60–63), was detected in a peptide extract derived from milk fermented

with an X-prolyl-dipeptidyl aminopeptidase (PepX)-deficient mutant of *Lact. helveticus* (Matar and Goulet 1996). However, the formation of casomorphins in fermented milk products due to LAB proteolytic activity seems unlikely due to the presence of PepX, which liberates X-Pro dipeptides from the N-terminus of peptides in several LAB strains (Christensen et al. 1999).

### 13.3.5. Bioactive Peptides Affecting Mineral Absorption

Milk is a rich dietary source of calcium, and its absorption may be enhanced when present in association with caseinophosphopeptides (CPP). CPP are a large group of peptides that have a phosphoserine residue in common. Phosphopeptides are released from  $\alpha_{s1}$ -,  $\alpha_{s2}$ -,  $\beta$ -, and  $\kappa$ -caseins by enzymatic hydrolysis either during fermentation or in the gastrointestinal tract. As a consequence of the high number of negative charges, these peptides efficiently bind divalent cations such as Ca, Mg, Fe, Zn, Cu, Mn, Ni, Co, Se, and Cr and, therefore, may act as biocarriers for these elements (Kitts and Weiler 2003). Calcium is a mineral of special concern because it has many important functions in the human body, including bone development and recalcification and the prevention of hypertension and colon cancer (FitzGerald 1998). While some controversy exists regarding CPP's ability to enhance *in vivo* Ca absorption, a number of reports involving animal studies have shown a positive effect of CPP on Ca absorption. The addition of CPP to Ca-fortified milks increased Ca absorption in growing rats (Tsuchita et al. 2001; Mora-Gutierrez et al. 2007).

The use of CPP for the prevention of dental caries has also been proposed because CPP are able to inhibit caries lesions through recalcification of the dental enamel (Oshiro et al. 2007). Recently, a CPP-stabilized amorphous calcium phosphate, Recaldent™ (Cadbury, UK), has become commercially available (Reynolds 1987, 2008). In addition, a protective effect of yogurt extract on dental enamel demineralization was demonstrated *in vitro* (Ferrazzano et al. 2008). Furthermore, CPP are used

as a supplement in bread, flour, cakes, beverages, and chewing gum.

Numerous phosphorylated peptides were identified in various cheese aqueous extracts or cheese juices at the end of the ripening (Grana, Emmentaler, Cheddar, etc.; Deutsch et al. 2000). Several CPP, consisting of a mixture of components derived from three parent peptides,  $\beta$ -casein f7–284P,  $\alpha_{s1}$ -casein f61–794P, and  $\alpha_{s2}$ -casein f7–214P, have been identified in a water-soluble fraction of Grana Padano cheese (Ferranti et al. 1997). Furthermore, some phosphopeptides were identified in the diafiltration retentate of a water-soluble fraction of Cheddar cheese (Singh et al. 1997), while nine phosphopeptides formed by the hydrolysis of  $\beta$ -casein were characterized in a soluble fraction of a Parmigiano-Reggiano cheese (Addeo et al. 1994). Phosphorylated peptides were more resistant than other peptides to hydrolysis by peptidases from thermophilic LAB, in particular to the aminopeptidases (Deutsch et al. 2000). Recently, mass-spectrometric screening of the main peptides generated by the action of the *Lact. delbrueckii* subsp. *lactis* CRL 581, a thermophilic lactic acid bacterium used for the manufacture of Argentinian homemade hard cheese, on  $\alpha$ - and  $\beta$ -caseins allowed the identification of several CPP in these hydrolysates (Hebert et al. 2008).

### 13.3.6. Antimicrobial Peptides

Antimicrobial peptides derived from food proteins constitute a new field in the use of antimicrobial agents in food (Clare et al. 2003; Hayes et al. 2007b). The biodefensive properties of milk have been recognized for many years. The antimicrobial activity of milk is attributed mainly to immunoglobulins, and to non-immune proteins, such as lactoferrin, lactoperoxidase, and lysozyme. These defense proteins can exert antimicrobial activities comparable to antibiotics, with potential application as natural alternatives (Hayes et al. 2007a).

We will now briefly summarize the main antimicrobial peptides derived from milk proteins. Lactoferricin (Lfcin) is perhaps the most well-known multifunctional peptide derived from lactoferrin. This peptide is generated by pepsin digestion

of lactoferrin. Lfcin elicits a more potent bactericidal effect than the parent molecule that may be due in part to its smaller size, which facilitates access to the target site on the microbial surface. Both bovine and human Lfcin display antimicrobial activity against a broad spectrum of microorganisms, including *Listeria monocytogenes* (Bellamy et al. 1992; Lopez-Exposito et al. 2008b). The antimicrobial activity of Lfcin against spoilage yeast or the pathogenic *Candida* has also been demonstrated (Wakabayashi et al. 2003; Enrique et al. 2008).

Although  $\alpha$ -lactalbumin and BLG have also been considered as potential precursors of bactericidal fragments, they present a narrowed spectrum of action, being active only against Gram (+) bacteria. Several antimicrobial peptides encoded in the primary structure of caseins have been described. Chymosin or milk-heating treatment of casein releases a group of basic, glycosylated polypeptides named casecidins (Lopez-Exposito et al. 2008a). They have been shown to display bactericidal properties against several pathogenic bacteria and lactobacilli. Isracidin, obtained from  $\alpha_{s1}$ -casein treated with chymosin at pH 6.4, corresponds to the N-terminal fragment of this protein (f1–23), show *in vivo* protection against lethal infections of *Staphylococcus aureus* in mice, and prevent mastitic infections in sheep and cow (Lahov and Regelson 1996). Casocidin-I (f150–188) is another prominent defense peptide that originates from  $\alpha_{s2}$ -casein. The fragments 164–179 and 183–207 from  $\alpha_{s2}$ -casein also show antimicrobial properties (Recio and Visser 1999; Lopez-Exposito et al. 2008a).

It has also been proposed that part of the antibacterial activity of human milk resides in glycosylated  $\kappa$ -casein. This type of antibacterial activity is related to the inhibition of the adhesion of the microorganism to the target tissue. For example, purified human  $\kappa$ -casein, in contrast to  $\kappa$ -casein purified from bovine milk, effectively inhibited the cell lineage-specific adhesion of fluoroisothiocyanate-labeled *Helicobacter pylori* to human gastric surface mucous cells (Stromqvist et al. 1995). Malkoski et al. (2001) also reported a direct bacterial growth-inhibition effect of  $\kappa$ -casein-derived peptide called kappacin, which corresponded to non-glycosylated,

phosphorylated bovine caseinomacropeptide f106–169.

Antimicrobial peptides derived from milk have also been reported following dairy fermentations. *Lact. acidophilus* DPC6026 was able to generate two peptide fragments from bovine casein that exhibited antimicrobial activity similar to that of the characterized antimicrobial peptide isracidin against pathogenic strains (Hayes et al. 2006). In addition, the partially purified proteinase from *Lact. helveticus* PR4 released the fragment 184–210 from  $\beta$ -casein that exhibited a broad spectrum of inhibition (Minervini et al. 2003). Finally, Rizzello et al. (2005) demonstrated antibacterial activity in two water-soluble extracts of nine Italian cheese varieties.

### 13.4. Analytical Strategies for the Characterization of Milk-derived Bioactive Peptides

In recent years, analytical research on milk bioactive peptides has followed three main directions aimed at developing and validating sensitive and specific methods for (1) tracing the formation pathway of bioactive peptides from the parent proteins; (2) identifying the bioactive peptides in milk and in derived foods; and (3) improving the “positive” properties discovered in natural peptides by designing of synthetic structural analogues or peptide mimetics. These studies aimed to clarify the structure-activity of peptides, which provides essential information for the design of novel therapeutics or functional food ingredients.

Until a few years ago, investigation has been limited to peptides obtained from *in vitro* digestion of proteins or on synthetic peptides. *In vivo* studies have been infrequently performed because of the enormous complexity of samples, which are, in most cases, composed of hundreds of structurally related peptides, especially in fermented dairy foods. However, most limitations have been overcome by the recent developments of mass spectrometry in the proteomic and peptidomic field. Today, the molecular weight of peptides and proteins may be accurately determined by electrospray MS (ESI-MS)

or matrix-assisted laser desorption ionization MS analysis. Furthermore, the mass spectrometer can be used to generate *de novo* amino acid sequence information, including post-translational modifications, from tandem mass spectra obtained either by post-source decay or by collision induced dissociation. Sample preparation is relatively simple, and MS analysis can be performed directly on the peptide extract. The peptides naturally present in human milk have been recently characterized (Picariello et al. 2008) using MS, which allowed the detection of possible bioactive sequences. The most powerful approach is obtained when the capability of MS in identifying protein structure is coupled to a high-resolution chromatographic or electrophoretic technique. More recently, ESI-MS, in combination with HPLC, has been used in an application to rationalize the pathway of casein breakdown by action of selected LAB strains, which increased enormously the amount of information on the production of possible bioactive sequences (Hebert et al. 2008).

The newest analytical approaches combine MS with cell biology, immunology, biochemistry, synthetic chemistry, and use of combinatorial library to identify the patterns of peptide formation and the bioactivity of the peptides present in the sample (Marasco et al. 2008). In milk-derived protein/peptides, identification of bioactive components can be accomplished as illustrated in Figure 13.3, following the general scheme: (1) purification of peptides present in a food sample (milk, yogurt, cheese) using high-resolution separation techniques or selective methods for enrichment of certain components (e.g., phosphopeptides); (2) peptide structural characterization by MS analysis of intact or enzymatically digested peptides; as a general feature, this peptidomic approach combines a high-resolution separation technique (two-dimensional gel electrophoresis, nano-HPLC) with MS or other microanalytical techniques for single protein identification, characterization of post-translational modifications, and database cataloguing; (3) *in silico* analysis for prediction of possible bioactive sequences; (4) synthesis of pure peptides, peptide analogues, or mimetics; and (5) confirmation of bioactivity through appropriate biological or cellular assays. In this

respect, the use of combinatorial chemistry to produce arrays of structurally related compounds for high-throughput screening is increasing the speed of discovery of drugs or functional ingredients (Koehn 2008).

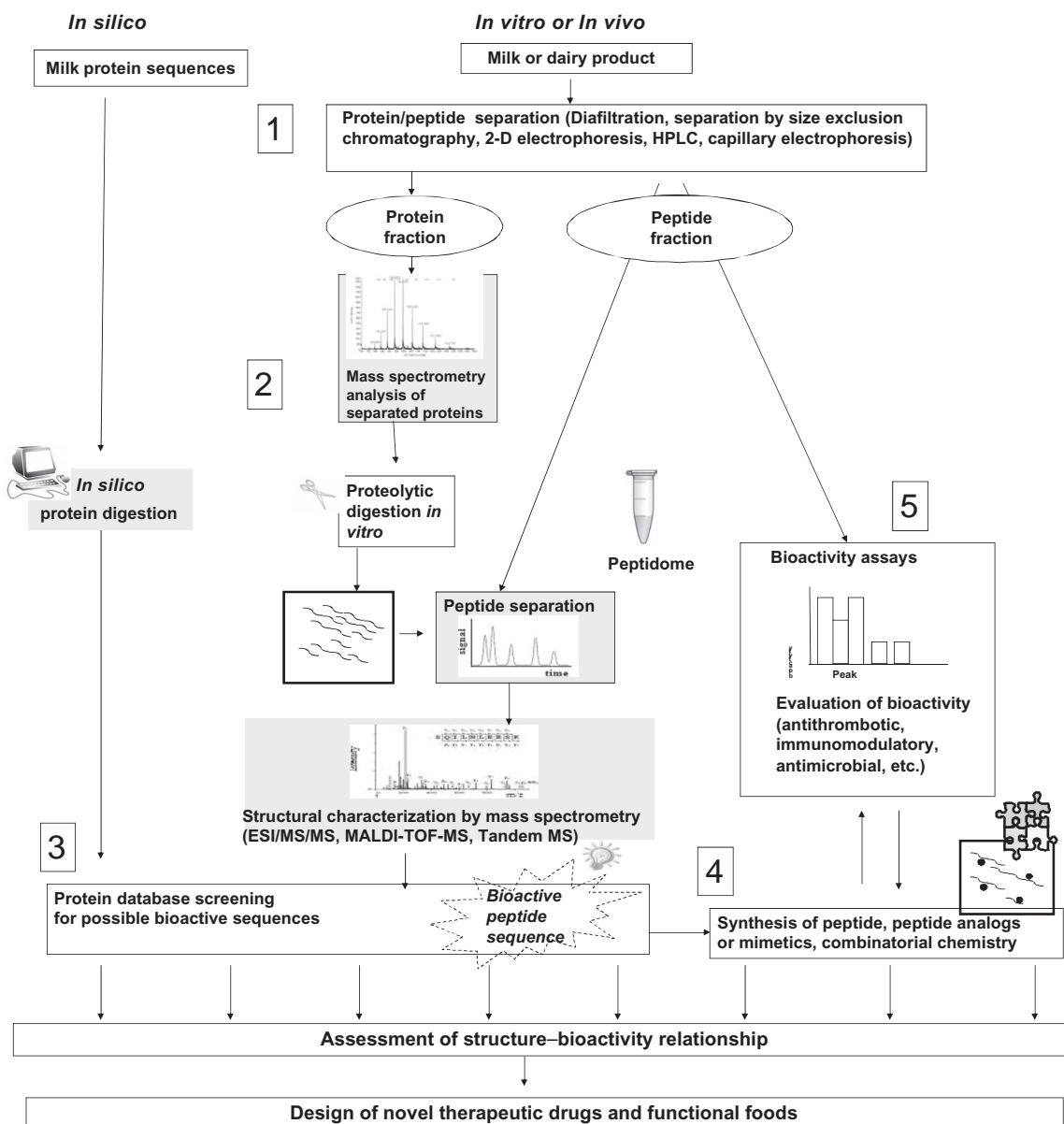
An illustrative example of the potential of these combined approaches is given by the characterization of peptides naturally released in whey during the production of Buffalo Mozzarella cheese (De Simone et al. 2009). MS structural identification of peptides, combined with the study of their antioxidant properties and immunomodulatory effects on intestinal cancer cells, suggested two candidate peptides for bioactivity, both derived from  $\beta$ -casein fragments, and their mechanism of action. In this respect, it seems reasonable to predict that the integration of the new and complementary analytical approaches illustrated above will provide a significant contribution to determining the structural features responsible for bioactivity, and the structure-function relationship of milk bioactive peptides.

### 13.5. Conclusions

The importance attributed to dairy proteins and bioactive peptides in nutrition and health is undeniable. Scientific evidence demonstrated that milk not only confers physiological benefits (essential vitamins, minerals, and macronutrients) but contributes with a variety of bioactive compounds with positive impact in human health.

In this chapter we described the main biological activities ascertained for milk-derived peptides such as antihypertensive, antithrombotic, immunomodulatory, opioid, antimicrobial, and mineral-binding properties. Although these peptides are inactive as part of the primary protein, they can be released and activated *in vivo* or *in vitro* by enzymatic proteolysis. For this reason, microbial fermentation especially by LAB, acquires a major role, since the production of key bioactive peptides can be triggered as foods are being produced. In this sense, the identification of new LAB strains capable of releasing milk-derived bioactive peptides and the understanding of the metabolic role of these compounds





**Figure 13.3.** Workflow analysis illustrating purification integrated strategies for the structural and functional characterization of milk bioactive peptides.

constitute the basis for the development of novel and improved functional foods.

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## Chapter 14

# New Approaches for the Study of Lactic Acid Bacteria Biodiversity: A Focus on Meat Ecosystems

Graciela M. Vignolo, Cecilia Fontana, and Pier S. Cocconcelli

*New molecular and genomic approaches are revealing a more dynamic and diverse view of the microbial world than originally anticipated. Culture-independent molecular methods are providing a better knowledge of the microbial biodiversity in a wide range of environments as well as establishing a link between function and complex relationships among the members of natural microbial consortiums. Microbial populations that develop in meat and meat products are the result of the prevailing environmental conditions on the growth of microorganisms initially present in raw materials or introduced by cross-contamination or processing. Intrinsic meat chemical factors, processing, and storage conditions determine the type and number of bacteria present in meat and meat products. The dynamics of lactic acid bacteria (LAB) and the composition of microbial communities resulting from the environmental determinants existing in raw chilled vacuum-packaged meat, fermented, and cooked meat ecosystems are discussed here. The understanding of how intermediary communities and individual LAB strains contribute to the bacteriological status of meat ecosystems is required for industrially useful innovations.*

### 14.1. Introduction

In the last decades, microbial ecology has undergone profound changes as a result of the huge impact of emerging technologies applied to the analysis of natural communities. Molecular and genomic analy-

ses revealed a vastly different view of microbial community ecology than was provided by traditional methods. The widespread use of molecular methods led to an increased ability to elucidate complex community relationships without the biases inherent to traditional culturing. In addition, a recent conceptual framework for using systematic, ecological, and evolutionary dynamics to identify the DNA sequence clusters corresponding most likely to the fundamental units of microbial diversity has been developed (Cohan and Perry 2007). By combining a population-based analysis of microbial diversity with the understanding of functional gene and protein expression, the implementation of “omics” technologies within integrated programs of environmental microbiology becomes crucial (Mullen et al. 2006; Nelson et al. 2007).

Food ecosystems constitute a large source of microbial diversity in which a number of bacterial communities coexist. Among these food ecosystems, meat ecosystems support the growth of highly specific microbial associations, the presence of which depend on factors that persist during processing, transportation, and storage; that is, many studies in meat microbiology have established that spoilage is caused by a dominating fraction of the initial microbial association in which high species diversity from a few bacterial genera was reported (Nychas et al. 2007). Consumers’ trust in meat as safe and wholesome is crucial. The major challenges for the meat industry in the 21st century are the control of traditional and emerging pathogens, food

additives, traceability, and the integrated management of meat safety risks applied from the producer to the consumer (Sofos 2008).

## 14.2. Biodiversity Establishment in Meat Ecosystems

The ecological strategies adopted by microorganisms to grow in meat ecosystems are the consequence of the prevailing environmental conditions, in which the intrinsic and extrinsic factors governing microbial growth will determine the type and number of bacteria present in meat, these factors being, respectively, predominantly chemical (concentration and availability of nutrients, pH, redox potential, buffering capacity,  $a_w$ , meat structure) and related to storage and processing conditions. The extrinsic factors (temperature and oxygen availability) are often manipulated to extend the shelf life of meat products. These physicochemical factors applied during meat storage are useful for explaining the selective action of the different factors outlined earlier on a complex microbiota, but they cannot explain why meat and meat products specifically select microorganisms that are not isolated from other food products.

The availability of nutrients in meat as a solid substrate and particularly the diffusion rate of fermentable compounds from the inner part of muscle to the surface limit bacterial growth to those especially featured organisms that are adapted to grow on this substrate (Gill and Penney 1977). A number of studies on meat microbiology showed that although the same bacterial genera have been reported among the dominating spoilage fraction, the findings at the species level were diverse (Nychas et al. 2007). The dominance of various species within a genus determines the range of microbial taxa found in meat under different processing and storage conditions. In the different meat ecosystems, the derived substances from the glycolytic pathway, oxidative products, and nitrogen energy sources such as glucose, lactate, pyruvate, and certain amino acids are catabolized by almost all meat-associated bacteria. As far as the role of proteolysis during meat aging is concerned, it was clearly shown that

the contribution of meat indigenous enzymes to its spoilage is negligible compared with the action of meat-associated bacteria (Nychas et al. 2008). The proteolytic activity of spoilage groups may gain an ecological advantage through meat penetration by having access to a new niche with newly available resources to be exploited, which would not be accessible to non- or less proteolytic bacteria (Gill and Penney 1977; Gupta and Nagamohini 1992). Meat spoilage can be considered an ecological phenomenon that encompasses the changes of the available substrates during the prevalence of a particular microbial association, or the so-called specific spoilage organisms. In fact, meat spoilage depends on an even smaller microbial fraction called “ephemeral spoilage organisms,” which is the consequence of factors that dynamically persist or are imposed during processing, transportation, and storage (Nychas et al. 2008).

Biodiversity is a measure of important ecological processes such as resource partitioning, competition, succession, and community productivity, as well as an indicator of community stability. Genetic variation among individuals within a population has long been recognized as the starting block for adaptation and evolution among microorganisms and other organisms. In general, the conditions under which bacteria must grow in meat as a substrate determine the different meat ecosystems. The microbial biodiversity of these ecosystems has received particular attention in areas where industrial applications are evident, such as in food biotechnology, and where microbial activity has important implications.

### 14.2.1. Chilled and Packaged Raw Meat Ecosystems

Skeletal muscles in all their forms constitute commercial commodities of strategic importance. Meat retailers, by breaking meat carcasses into retail cuts, give rise to the first meat ecosystem in the meat processing chain. Systems for retail meat distribution and commercialization are principally based on vacuum-packaging (VP) or modified atmosphere packaging (MAP) of meat cuts using low gas perme-

ability films and refrigeration. These methods, based on the use of gas mixtures containing variable O<sub>2</sub> and CO<sub>2</sub> concentrations, have proved very effective in extending the shelf life of perishable foods, such as muscle foods, and preventing the growth of food-borne pathogens (Belcher 2006; McMillin 2008). Moreover, considering that most of the meat consumed in developed countries is produced in developing countries, centralized packaging systems for the distribution of retail-ready meat were shown to maximize storage life during meat transport to distant markets (Jeyamkondan et al. 2000; Belcher 2006). Substantial fractions of CO<sub>2</sub> are used to retard the growth of aerobic spoilers, and a certain concentration of O<sub>2</sub> is employed for red meat color preservation. Depending on the gas permeability of the packaging material, O<sub>2</sub> supply will be restricted, and thus a selective effect on the microbial population is produced (Labadie 1999).

After slaughter, microbial contamination of carcasses is the consequence of the conditions of animal rearing and feeding, transport logistics, lairage, and slaughter as well as the methods applied to meat conditioning, these procedures influencing not only the quantity of bacteria but also the type of microorganisms present (Fegan et al. 2004; Arthur et al. 2007). Although some differences exist between animal species in the type of microorganisms isolated from the carcass, generally the same bacterial species can be isolated from beef, pork, sheep, and even chicken carcasses (Labadie 1999). The microorganisms present in meat originate in the animal intestinal tract as well as in the environment with which the animal had contact before or during slaughter (Koutsoumanis and Sofos 2004). Meat is a selective agent for aerobic microbiota, and a consortium of bacteria commonly dominated by *Pseudomonas* spp. is responsible for the spoilage of meat stored aerobically at temperatures between -1 and 25°C. Cold-tolerant Enterobacteriaceae, *Brochothrix thermosphacta*, and lactic acid bacteria (LAB) also occur in chilled meat stored aerobically, but in terms of numbers they do not contribute to dominating microbial associations (Nychas et al. 2008). Both LAB and *B. thermosphacta*, the most important causes of spoilage commonly associated

with meat packed under VP and MAP conditions, result from the competition between facultative anaerobic Gram (+) biota. However, some special features of ephemeral spoilers may be correlated with their rapid growth on meat as a substrate. *Pseudomonas fragi*, the fastest aerobically growing species in meat at low temperatures, does not synthesize the siderophore pyoverdinin necessary for its aerobic metabolism, a property that probably saves energy for the bacterial cell; because this bacterium is able to utilize siderophores of different origins, its iron requirement is fulfilled from the different sources available in the meat, including hemoglobin (Champomier-Vergés et al. 1996). In addition, proteases from *Ps. fragi* are secreted inside bleb-off structures, which allow penetration into the meat, facilitating its invasion and protein degradation (Thompson et al. 1985). These adaptation mechanisms of *Ps. fragi* may explain the predominance of this bacterium during chill aerobic meat storage.

On the other hand, the psychrotrophic *B. thermosphacta* frequently represents a significant portion of the spoilage microbiota of aerobic, VP, and MAP meats firstly influenced by low temperatures and the proportion of *B. thermosphacta* in the initial biota. However, these factors do not explain why *B. thermosphacta* can be isolated only from slaughterhouses, chilling facilities, meat, and meat products. The interaction of *B. thermosphacta* with other meat spoilage microbial groups showed a decrease in the growth of this bacterium at 5°C in the presence of LAB, while it was the dominant organism in the presence of *Pseudomonas*, LAB, and Enterobacteriaceae (Russo et al. 2006). Recently, analysis of the 1.8Mb genome sequence of *Lactobacillus sakei*, a meat-borne lactic acid bacterium, suggested the evolution of this organism to adapt itself to grow and survive on meat and meat products (Chaillou et al. 2005). The adaptation to meat, an environment rich in amino acids as a result of endogenous proteases activity, leads to a lack of biosynthetic pathways for amino acid syntheses, *Lact. sakei* being auxotrophic for all amino acids but not for aspartic and glutamic acids (Champomier-Vergés et al. 2002). After glucose depletion in meat, the specialized metabolic repertoire of this organism

includes purine nucleoside scavenging as well as arginine degrading ability, which provides an alternative energy source, facilitating *Lact. sakei* growth in meat. Despite a predilection for anaerobiosis, a versatile redox metabolism allows *Lact. sakei* to deal surprisingly well with oxidative stresses, iron and heme acquisition being somehow correlated with such resistance. Biofilm formation and cellular aggregation also appear to be mechanisms for *Lact. sakei* to compete during meat surface colonization, overcoming nutrients fluctuations (Chaillou et al. 2005). Moreover, this species has psychrotrophic and osmotolerant properties, and is able to grow at low temperatures and in the presence of up to 10% of NaCl, these features being associated with the presence in the *Lact. sakei* genome of a number of genes coding for stress response proteins (Marceau et al. 2004). As for *Lact. sakei*, a combination of factors is likely to play a role in the strong growth of *Leuconostoc* spp. and *Carnobacterium* spp. in meat. In particular, *Carnobacterium* is tolerant to freezing/thawing and can grow anaerobically with increased CO<sub>2</sub> concentrations and at temperatures as low as 2 to −1.5°C (Leisner et al. 2007). Nevertheless, chill temperatures and oxygen concentration are the most selective parameters that determine the specific microbiota that is able to grow in this ecological niche.

#### 14.2.2. Fermented Meat Ecosystem

Fermentation and drying can be considered as the oldest ways of preserving raw materials. Although the historical origin of fermented meat products remains unknown, fragmentary bibliographical research has traced it back more than 2500 years in China (Zhou and Zhao 2007). Many fermented meat products have been known in Europe since the 13th–14th centuries after being introduced by Marco Polo. Proof of sausage production was first documented in ancient Greece, where it may have been propitiated by the existing climate conditions (Liepe 1983). This tradition was inherited by the Romans, and from then on fermented sausages spread to Central, Eastern, and Northern European countries as well as to America and Australia, where it is

recognized as a European heritage (Demeyer 2004; Fadda and Vignolo 2007).

Fermented sausages can be defined as meat products consisting of a mixture of mainly meat, fat, salt, curing agents, and spices stuffed into casings, fermented (ripened), and dried. They are prepared from a wide range of meat species and different production technologies, which account for the existence of a large variety of fermented sausages. Meat from adult animals is preferred because of its higher myoglobin content, which favors stable color formation. The functional characteristics of meat, such as composition, pH, and binding properties, are major criteria when selecting meat, while fat (10%–40%) should be firm, white, and fresh with a high melting point and a low polyunsaturated fatty acid content (Demeyer 2004; Lebert et al. 2007). Among additives, NaCl is normally added at levels ranging from 2% to 4%, depending on technology and market demands, while nitrite and/or nitrate are used at a level of 150–250 ppm, also depending on the meat product and on country regulations (Honikel 2008). Sugars are commonly added, dextrose, glucose, sucrose, and lactose being the most often used as well as corn syrup and different starches, whose main role is to act as substrates for LAB to produce lactic acid and decreased pH. Spices are the main factor that differentiates fermented sausages, pepper, paprika, and/or garlic from others that are currently used. The manufacture of dry fermented sausages is considered to occur in three phases: mixing meat with pork fat, salt, sugars, nitrate/nitrite, and spices; fermentation; and ripening/drying, which do not always constitute two separate steps (Lebert et al. 2007). During fermentation, two microbial reactions occur simultaneously and interdependently: the decrease in the pH through glycolysis by LAB and the production of nitric oxide by nitrate- and nitrite-reducing bacteria such as coagulase-negative cocci (CNC) involving *Staphylococcus* and/or *Micrococcus*.

Microorganisms that are present in fermented and cured meat ecosystems have their origin in the raw materials used in their production as well as in the fermentation and drying conditions. The presence of ecological determinants influences the



establishment of a specific microbial association that will determine the rate of colonization. Sodium chloride, nitrate/nitrite, sugars, and the presence of  $a_w$  (0.85–0.92), temperature (24–30°C to 12–18°C), and oxygen gradients during ripening will highly select the microbiota that is able to develop in this ecological niche. In traditional fermented sausages, *Lact. sakei*, *Lactobacillus curvatus*, *Lactobacillus plantarum*, and *Pediococcus* spp. are by far the most often isolated species among LAB, which must have adapted to the existing stringent conditions. Although *Lact. plantarum* is a versatile bacterium with a genome size of 3.3 Mb (Kleerebezem et al. 2003), and it has been identified as part of the fermented sausages microbiota, this species lacks the meat specialization found in *Lact. sakei*. Less information is available on the physiology and genetics of *Lact. curvatus* other than the production of antibacterial substances, which is an effective and widespread bacterial tool for microbial competition among LAB.

On the other hand, CNC in fermented sausages and dry-cured hams originate from meat, human contact, and salt (Cordero and Zumalacárregui 2000), and their growth is promoted by their ability to survive environmental stresses such as high salt content and low temperatures in meat fermentation. Many CNC from the genus *Staphylococcus* were isolated from these ecosystems; *Staphylococcus xylosus*, whose genome sequencing is ongoing (Dordet-Frisoni et al. 2007), has been shown to be very competitive during the fermentation of dry and semidry sausages. Apart from LAB, CNC, mold, and yeasts, which are involved in meat fermentation, pathogenic bacteria, introduced by meat and casings, may also grow. The existence of a series of hurdles either specifically included (NaCl,  $\text{NO}_2/\text{NO}_3$ , ascorbate) or indirectly created in the stuffed mix (low reduction potential, pH, and  $a_w$ , LAB bacteriocins) will regulate bacterial growth in this ecosystem.

#### 14.2.3. Cooked Meat Ecosystem

Inactivation of bacterial growth by high temperatures has been used as an effective food preservation tool since Pasteur in the 19th century. Meat pro-

cessed (cured or not) products that have been subjected to a thermal process and are frequently commercialized refrigerated and under VP or MAP conditions constitute this ecosystem. Cooked, cured or not, meat products are economically important ready-to-eat commodities, including cooked ham, emulsion-style sausages, smoked pork loin, bacon, pâté, and cooked turkey/poultry. As these meat products are heated to a temperature of 65–75°C, most vegetative cells are killed, and post-heat treatment recontamination determines their shelf life (Vermeiren et al. 2005). The microbial ecosystem of cooked meat products is determined by product composition and handling after cooking, fluctuations within the cold chain, hygienic conditions throughout the processing line, and storage as well as mishandling domestic practices (Samelis et al. 1998, 2000; Marklinder et al. 2004). Anaerobic bacterial spoilage is linked to the production of acids, off-flavors, and color-deteriorating compounds, often leading to product rejection. Under these conditions, a selection is produced for a spoilage microbiota of LAB and *B. thermosphacta* (Cayré et al. 2005), which are initially present in low numbers as ephemeral spoilage organisms (Nychas and Skandamis 2005). *Lactobacillus*, *Carnobacterium*, *Leuconostoc*, and *Weissella* frequently become the dominant LAB in cooked meat ecosystems (Samelis et al. 2000; Santos et al. 2005; Vermeiren et al. 2005; Vasilopoulos et al. 2008). In addition to the presence of LAB, the spoilage organism *B. thermosphacta* is found, in particular if oxygen is present in the package (Cayré et al. 2005), while the psychrotrophic pathogen *Listeria monocytogenes* may also be present, mainly as a result of post-contamination.

### 14.3. Experimental Approaches to the Study of Microbial Ecology in Meat Ecosystems

#### 14.3.1. The Polyphasic Approach in LAB Taxonomy

The importance of ecological concepts in understanding the presence and growth of microorganisms in foods is now being recognized. The

production of fermented foods under controlled conditions, and their safety assurance depend on the knowledge and control of their microbiota. Traditional fermented meat products are obtained by spontaneous fermentations (in which no inocula are added) and contain complex microbiota, which are difficult to describe through traditional microbiological methods. The microbial structure of these foods can be studied using different approaches. The typification of “isolated microorganisms” with methods based on a combination of phenotypic (physiological and biochemical) characteristics known as “traditional” methods and genotypic identification techniques constitute the so-called culture-dependent methodology. Many genotypic methods are based on the principle of the polymerase chain reaction (PCR) and exhibit various levels of discriminatory power, from species level to individual strains. Some of the most often used DNA-based methods in LAB taxonomy are species-specific PCR (s-s PCR), Restriction fragment length polymorphism (RFLP) or ribotyping, amplified fragment length polymorphism (AFLP), amplified ribosomal DNA restriction analysis, pulsed-field gel electrophoresis (PFGE), and random amplified polymorphic DNA (RAPD). On the other hand, to eliminate problems related to traditional isolation and to detect microorganisms that are not culturable or not yet cultured, nucleic acids are directly extracted from the food matrix and the microbial diversity is determined without the need for prior cultivation. Culture-independent genomic or “metagenomic” surveys of microbial communities in their natural environments are carried out using techniques such as PCR denaturing gradient gel electrophoresis (PCR-DGGE), PCR temperature gradient gel electrophoresis (PCR-TGGE), and fluorescence *in situ* hybridization (FISH).

During the last decade, the genome of the most important LAB has been sequenced (Klaenhammer et al. 2002; Kleerebezem et al. 2003; Chaillou et al. 2005), which can help in the design of appropriate primers and probes for the molecular identification, control, and monitor of LAB species in meat ecosystems. On the other hand, the development of a real-time PCR or quantitative PCR (Q-PCR), a

molecular culture-independent enumeration method, was shown to be particularly valuable to enumerating LAB in complex microbial communities. This method allows an accurate and unambiguous identification and quantification of nucleic acid sequences (Klein 2002), and, compared with conventional PCR, cross-contamination is reduced while high throughput, wider dynamic range, and automation can be achieved.

#### 14.3.2. Culture-dependent Methods

Culture-dependent techniques involve the traditional cultivation methods on synthetic media resembling the conditions of the ecosystem from which the microorganisms are isolated, in combination with phenotypic and genotypic identification techniques. These studies, based on culturing techniques, are laborious and time-consuming, in many instances requiring 8–10 days to be completed. Moreover, such studies have focused commonly on the analysis of end-products. A first problem in the characterization of the microbiota of meat products by bacteriological culturing is the selection of colonies in the plates. Generally, colonies are randomly selected, but they may not represent the real microbiota composition. In this sense, a large number of colonies must be selected, purified, and identified to the species level by phenotypic or genotypic identification methods. Although physiological, nutritional, and biochemical tests such as the API® system (BioMérieux, La Balme les Grottes, France), MicroLog® (Biolog Inc., Hayward, CA), and Diatabs™ (Rosco, Taastrup, Denmark) are time-consuming and somewhat difficult to standardize, these traditional tests still have great importance in taxonomy. Identification keys for most of these tests are not accurate with LAB species, but the assays themselves are valuable as a convenient way to carry out large-scale phenotypical studies. Based on this, a polyphasic or combined approach using genotypic characterization techniques is preferred. The application of tools based on molecular methods to study changes in the microbiota composition of the different meat ecosystems allowed the fast and unequivocal identification of the isolated strains.

The major categories of bacterial typing techniques as well as their general concept and applications to study bacteria from meat ecosystems at different taxonomic levels will be roughly described.

*Typing of isolated microorganisms.* Genotypic techniques exhibit various levels of discriminatory power, from the species level to the differentiation of individual strains (typing). The genotypic methods based on the PCR principle enables the selective amplification of specifically targeted DNA fragments by using oligonucleotide primers under controlled reaction conditions. PCR primers can be designed for amplification at any taxonomic level. The PCR-based detection of LAB and other bacteria mostly requires a difficult design strategy and thorough validation before a valuable set of primers is obtained. The increasing availability of the sequences of the 16S rRNA gene (Collins et al. 1991) and the intergenic region between 16S rRNA and 23S rRNA genes (Nour 1998) allowed the development of different methods for identifying microbial species of interest in the field of meat and meat products. Among these methods, ribosomal RNA probes (Hertel et al. 1991; Nissen and Dainty 1995) and s-s PCR primers (Berthier and Ehrlich 1998; Yost and Nattress 2000; Rossi et al. 2001; Blaiotta et al. 2003) have been applied.

As a relatively small molecule, the 16S rRNA encoding gene can be sequenced directly, without laborious cloning, from PCR amplicons created with universal 16S primers. The 16S/23S rRNA intergenic spacer region has been employed as a DNA-specific probe for the identification of LAB (Hensieck et al. 1992; Hertel et al. 1993; Ehrmann et al. 1994; Frahm et al. 1998; Guarnieri et al. 2001). The same target sequences have been used as primers for the detection of CNC by s-s PCR (Forsman et al. 1997; Gory et al. 1999). The variations in length and sequence of the 16S-23S rRNA intergenic spacer regions have proved useful for the identification of strains and species (Barry et al. 1991; Jensen et al. 1993; Gürtler and Stanisich 1996; Berthier and Ehrlich 1999); the evolutionary rate of the region is 10 times greater than that of 16S rRNA, allowing closely related bacterial species to

be distinguished (Gürtler and Stanisich 1996). The s-s PCR assay using oligonucleotides designed on the basis of 16S rRNA genes may be a useful approach; however, it is not suitable for some species because of the high identity value of their 16S ribosomal DNA sequences (Collins et al. 1991). In this case, the *recA* gene may be used as a phylogenetic marker (Eisen 1995; Lloyd and Sharp 1993).

In addition, to rapidly detect multiple bacteria in a single reaction, multiplex PCR has been developed (Chamberlain et al. 1988). Simultaneous amplification of more than one locus is required to allow the rapid identification of several isolates. It also constitutes a fast culture-independent approach for species-specific detection in complex matrices, being useful in defining the structure of certain microbial consortiums, and in evaluating community dynamics during fermentation or as response to environmental variations. The application of this technique in the identification of microorganisms transported through foods and beverages was recently reviewed by Settanni and Corsetti (2007).

*DNA profiling methods.* Total DNA or amplicons resulting from a selective PCR reaction may be digested by restriction enzymes, resulting in a mixture of fragments of different sizes. This technique is commonly referred to as RFLP and is the prototype of a DNA fingerprinting method mostly used in identifying isolates at the intraspecific level. In 1986, Grimont and Grimont reported the 16S and 23S rRNA RFLP method or ribotyping that combines an enzymatic restriction digest with the detection of the resulting fragments by means of rDNA probes. To a large extent, the discriminatory power of this technique depends on the number and type of restriction enzymes and probes used. In the past few years, ribotyping has been used in taxonomic studies for *Lactobacillus*, *Streptococcus*, *Leuconostoc*, and *Weissella* species or subspecies (Schlegel et al. 2000; Björkroth et al. 2000, 2002; Fernández et al. 2004; Suzuki et al. 2004; Kostinek et al. 2005).

Another combination of the PCR and restriction enzyme methodologies yielded the AFLP technique (Janssen et al. 1996), whose basic principle is RFLP

analysis but with a PCR-mediated amplification to select particular DNA fragments from the pool of restriction fragments. Restriction is performed by using two restriction enzymes that yield DNA fragments with two different randomly combined types of sticky ends to which short oligonucleotides (adapters) are ligated to form templates for the PCR. The selective amplification reaction is performed by using two different primers containing the same sequence as the adapters but extended to include one or more selective bases next to the restriction site of the primer. Only fragments that completely match the primer sequence are amplified. An array of about 30 to 40 DNA fragments are amplified, some of them being group-specific and others being strain-specific (Janssen et al. 1996). Within LAB, species-level discrimination has been documented for the phylogenetically closely related species *Lactobacillus pentosus*, *Lactobacillus pseudoplantarum*, and *Lact. plantarum* (Giraffa and Neviani 2000).

DNA fingerprinting techniques that rely solely on PCR include RAPD (Dellaglio et al. 2005; Valcheva et al. 2005) and repetitive genomic element (rep)-PCR (Gevers et al. 2001; Kostinek et al. 2005; Švec et al. 2005a, 2005b). RAPD analysis utilizes short arbitrary primers and low-stringency conditions to randomly amplify DNA fragments, which are separated electrophoretically to produce a fingerprint. This method offers great flexibility in primer choice; thus, it can be used to differentiate LAB at different taxonomic levels, ranging from genus to intraspecific level. In contrast, (rep)-PCR-specific primers amplify repetitive bacterial DNA elements such as ERIC, BOX, or (GTG)<sub>5</sub> (Versalovic et al. 1991). In recent studies, (GTG)<sub>5</sub>-PCR proved useful for the differentiation of lactobacilli and bifidobacteria at species, subspecies, and potentially strain levels (Hyytiä-Trees et al. 1999; Gevers et al. 2001; Masco et al. 2003; Cocolin et al. 2004a). PFGE of restriction endonuclease-digested genomic DNA is a powerful genotyping method for bacterial strain characterization due to its high resolution and excellent reproducibility (Tenover et al. 1995; Björkroth et al. 1996; Ventura and Zink 2002). PFGE employs an alternating field of electrophoresis to allow sepa-

ration of the large DNA fragments obtained from restriction digests with rare-cutting enzymes, in which the extraction of intact chromosomal DNA is crucial. Although PFGE is highly reliable and has a superior discriminatory power with excellent sub-species differentiation for a large number of microorganisms (McCartney 2002), it is time-consuming (4–5 days) and is not suited for routine assays.

In addition, numerical analysis of protein patterns created by highly standardized SDS-PAGE also revealed a valuable method for the investigation of bacterial (including LAB) relationships at the genus or species level (Schleifer and Stackebrandt 1983; Pot et al. 1994; Dicks 1995; Vandamme et al. 1996). Even when this method is not based on PCR reaction, SDS-PAGE whole-cell protein profiling (WCPP) was used to identify certain LAB species and subspecies (Björkroth and Holzapfel 2003; Hammes and Hertel 2003).

#### 14.3.3. Culture-independent Methods

The identification methods described in the previous section rely on the ability to isolate and cultivate LAB from different food or environmental samples. Because these culture-dependent approaches have shown limitations in terms of recovery rate, the set of obtained isolates may not always truly reflect the microbial composition of the sample (Ampe et al. 1999; Ercolini et al. 2001). As a result, culture-independent methods have been developed to circumvent the limitations of conventional cultivation for analysis of microbial communities (Vaughan et al. 2002). This approach eliminates problems related to selective cultivation and isolation of bacteria from natural samples. The main reason for the use of culture-independent techniques is the lack of knowledge of the real conditions under which most bacteria grow in their natural habitat and the difficulties in developing cultivation media that accurately resemble these conditions.

Genetic fingerprinting techniques are able to provide a profile representing the genetic diversity of a microbial community from a specific environment. PCR-DGGE and PCR-TGGE are routinely

used worldwide to study microbial communities and population dynamics in microbial ecology. These two techniques essentially consist of the amplification of the genes encoding the 16S rRNA from the matrix containing different bacterial populations, followed by the separation of the DNA fragments. Separation is based on the decreased electrophoretic mobility of PCR-amplified, partially melted, double-stranded DNA molecules in polyacrylamide gels containing a linear gradient of DNA denaturants (DGGE), or a linear temperature gradient (TGGE). Molecules with different sequences may have different melting behaviors and will stop migrating at different positions along the gel. DGGE is perhaps the most applied culture-independent fingerprinting technique, and, by adjusting the primers used for amplification, both major and minor constituents of microbial communities can be characterized. Different authors (Muyzer and Smalla 1998; Muyzer 1999; Ercolini 2004) have reviewed the application of DGGE in the field of microbial ecology. At present, DGGE has been widely applied in various research areas, including the detection of LAB in food samples and other environments. Additionally, DGGE supports the species identification of community members because after separation of the amplification products by DGGE they can be recovered from the gels and sequenced. The combination of DGGE analysis with reverse transcriptase (RT)-PCR has been described to study both the microbial composition and the metabolic activity during food manufacturing (Coccolin et al. 2001, 2004a; Randazzo et al. 2002).

The fastest culture-independent approach for genus-, species-, or strain-specific detection of LAB in a food matrix is the use of specific primers for the PCR-based detection of the target organisms in the bacterial DNA pool extracted from the sample. Probing techniques are based on the hybridization of synthetically designed oligonucleotides to specific target sequences in bacterial DNA. Unlike conventional PCR primers, these probes are linked to a radioactive or fluorescent label, which enables the visual detection of the target after hybridization under controlled conditions. The specificity of the

probe is largely dependent on the target sequence, although the stringency of the hybridization and washing conditions are also critical (O'Sullivan 1999). Labeled oligonucleotide probes are employed in a number of assays, including colony, dotblot, and *in situ* hybridizations (Giraffa and Neviani 2000). The most frequently applied method using probes is FISH, which uses fluorescence microscopy for the counting of fluorescently labeled bacteria. Despite considerable background knowledge of FISH, application of this method to the study of microbial population distribution in food has been limited (Ercolini et al. 2003; Coccolin et al. 2007).

Reliable and fast identification methods are of great importance to control and monitor either endogenous or inoculated starter cultures. The development of a molecular culture-independent enumeration method appears to be particularly valuable in the case of LAB since other methods are not efficient to enumerate this bacterial group in complex microbial communities. The enumeration of LAB species is important to the study of their role and dynamics in various niches. Quantitative PCR allowed the specific counting of bacterial cells in a LAB species or group of LAB species present in fermented products: *Lactobacillus* species in dental caries (Byun et al. 2004), *Lactobacillus thermotolerans* in chicken feces (Selim et al. 2005), *Enterococcus* in environmental samples (He and Jiang 2005), *Lactococcus lactis* subsp. *cremoris* in fermented milk (Grattepanche et al. 2005), and LAB species in milk products (Furet et al. 2004). The precision of molecular quantification by Q-PCR depends on the effectiveness of bacterial lysis and of DNA extraction, and on the presence of PCR inhibitors in the DNA solutions (Ludwig and Schleifer 2000; Hein et al. 2001). Martin et al. (2006) reported a rapid and sensitive Q-PCR assay for reliable quantitative identification and monitoring of *Lact. sakei* during sausage fermentation, both from endogenous microflora or inoculated starter cultures, and for distribution and abundance studies of this bacterium in complex microbial communities.



## 14.4. Meat Ecosystems Microbial Diversity: A Focus on LAB

### 14.4.1. Raw Meat Stored under VP and MAP Conditions

Due to the economic importance of meat, the microbial ecology of VP and MAP raw meat has been extensively studied. Table 14.1 shows the major LAB species isolated from VP beef from different geographic regions in which a dominance of *Lactobacillus*, *Leuconostoc*, and *Carnobacterium* has been observed. Using traditional methods, Schillinger and Lücke (1986) found only psychrotrophic LAB in VP beef stored at 2°C, among which *Lact. sakei*, *Lact. curvatus*, *Lactobacillus carnis*, and *Lactobacillus divergens* (further classified as *Carnobacterium divergens*) were identified as the predominant microbiota. Later, McMullen and Stiles (1993) reported the influence of storage temperature (−1, 4.4 and 10°C) on the type of bacteria dominating the microbial population of fresh pork stored under MAP. Even when homofermentative

lactobacilli biodiversity was greater at 4.4 and 10°C, *Lact. sakei*, *Lactobacillus alimentarius*, and *Lactobacillus farciminis* were present at all three assayed temperatures. In addition, species of LAB was shown to depend on the growth medium; the majority of isolates from plate count agar were carnobacteria, whereas those from MRS agar were homofermentative LAB, and *Leuconostoc gelidum* was isolated from both culture media. When DNA-based methods were used to characterize the microbiota composition of chilled VP beef, a lactobacilli population in which *Lact. sakei* and *Lact. curvatus* prevailed was found in most of the studies using either culture-dependent (Yost and Nattress 2002) or culture-independent methods (Fontana et al. 2006; Ercolini et al. 2006). Moreover, *Leuconostoc* species were also identified using RAPD-PCR techniques; *Leuc. gelidum* and *Leuconostoc carnosum* were identified by multiplex PCR as were other relevant populations in Argentinean beef stored both at 2 and 8°C under VP conditions (Fontana et al. 2006). When different environmental conditions

**Table 14.1.** Major LAB species isolated from VP and MAP meat.

	Identification method	Source	References
<i>Lact. sakei/curvatus/divergens/ carnis</i> ; <i>C. piscicola</i> ; <i>L. raffinolactis</i> ; <i>Leuconostoc</i> sp.	Traditional	VP beef; 2°C/32 days (Germany)	Schillinger and Lücke (1986)
<i>Lact. sakei/curvatus/alimentarius/ casei/farciminis/coryneformis</i> ; <i>Leuc. gelidum/mesenteroides</i>	Traditional	VP pork; −1.4 and 4.4°C/10 weeks; 10°C/4 weeks (Canada)	McMullen and Stiles (1993)
<i>Pediococcus</i> sp.; <i>C. divergens</i>	SDS-PAGE; WCPP	VP beef; 2°C/6 weeks (Japan)	Sakala et al. (2002)
<i>Lact. algidus</i> ; <i>L. piscium</i> ; <i>Leuc. gelidum</i> ; <i>C. piscicola/divergens</i>			
<i>Lact. sakei/curvatus</i> ; <i>Leuconostoc</i> sp.	RAPD-PCR; 16S DNA seq	VP beef; 2°C/6 weeks (Canada)	Yost and Nattress (2002)
<i>Lact. delbruekii</i> ; <i>C. divergens</i> ; <i>Leuc. mesenteroides</i>	PFGE	VP beef; −1.5°C/16 weeks (New Zealand)	Jones (2004)
<i>Lact. sakei/curvatus</i> ; <i>Leuc. carnosum/gelidum</i>	PCR-DGGE; S-s PCR; M-PCR	VP beef; 2°C/ 9 weeks; 8°C/14 days (Argentina)	Fontana et al. (2006)
<i>Lact. sakei/graminis</i> ; <i>Leuc. kimchii/carnosum</i> ; <i>C. divergens</i> ; <i>W. hellenica</i>	PCR-DGGE	MAP (20/60% O <sub>2</sub> ; 40% CO <sub>2</sub> ) beef; 5°C/2 weeks (Italy)	Ercolini et al. (2006)
<i>Lact. algidus/sakei</i> ; <i>Leuc. gasicomitatum/gelidum</i> ; <i>C. divergens</i>	RFLP	MAP (70% O <sub>2</sub> ; 30% CO <sub>2</sub> ) beef; 0–2°C/4 weeks (Finland)	Vihavainen and Björkroth (2007)

*Lact* = *Lactobacillus*; *L* = *Lactococcus*; *C* = *Carnobacterium*; *Leuc* = *Leuconostoc*; *W* = *Weissella*.

were assayed (Ercolini et al. 2006), spoilage was shown to occur between 7 and 14 days of storage at 5°C, with LAB species found in beef varying according to packaging conditions. *C. divergens*, *Weissella hellenica*, and *Lactobacillus graminis* were identified as acting during storage in air, while *Lact. sakei* was reported to be always present in beef stored under both MAP conditions containing 60% or 20% oxygen at any sampling time. However, at certain sampling times *Leuconostoc kimchii* and *Leuc. carnosum* were also found under MAP conditions. When SDS-PAGE WCPP in combination with biochemical and physiological characteristics was used to follow the changes in the microbiota of VP beef stored at 2°C, *Lactobacillus algidus*, *Lactococcus piscium*, *Leuc. gelidum*, and *C. divergens* were identified as the dominant LAB (Sakala et al. 2002). On the other hand, PFGE and biochemical analysis enabled the determination of a pattern of LAB succession during VP beef storage at -1.5°C in which the transition of *C. divergens* to *Leuconostoc mesenteroides* dominance was determined in association with pH dropping to below 5.4; this decrease was observed to be a driver of succession in these populations (Jones 2004). Although *Lact. sakei* was described as the dominant *Lactobacillus* species present in VP or MAP beef stored at low temperatures, it was not established when SDS-PAGE WCPP and PFGE techniques were applied. Instead, *L. piscium* and *Lactobacillus delbrueckii* were reported to be present using these methods, respectively; lactococci were rarely isolated from VP or MAP beef, except in the studies of Schillinger and Lücke (1986) and Sakala et al. (2002). Recently, the predominant LAB in spoiled value-added, high-oxygen MAP beef steak products were identified by Vihavainen and Björkroth (2007) using RFLP. *Leuconostoc gasicomitatum*, *Leuc. gelidum*, *Lact. algidus*, *Lact. sakei*, and *C. divergens* were found to be the dominant species, with the *Leuconostoc* species responsible for green discoloration and buttery off-odors.

The dominance of LAB in VP meat occurs within a relatively broad set of conditions, which coincide with those used in commercial storage. Among LAB, *Lact. sakei* and *Leuc. gelidum* dominate, while

other species grow to constitute a minor part of the microbiota. The benefit of LAB during the storage of raw meat is quite controversial. Although LAB are recognized as causative agents of meat spoilage (Jones 2004; Koutsoumanis et al. 2006; Vihavainen and Björkroth 2007), the selective growth promotion of these bacteria by capitalizing on their ability to control meat-borne pathogens and contaminants, resulting in a preferential growth of benign strains, should minimize their spoilage effects.

#### 14.4.2. Microbial Diversity of Fermented Sausages

The microbiota that participates in the fermentation and ripening of sausages originates in the environment, the equipment, and the raw material used in the manufacture of the products, and is highly dependent on the technology used. To our knowledge, the first studies on the ecology of fermented sausages date back to the 1960s (Lerche and Reuter 1960). Since then, LAB, mainly *Lactobacillus* and CNC, represented by Micrococcaceae, have been recognized as the two main groups of microorganisms that are considered technologically important in the fermentation and ripening of sausages. The information existing on the ecology of fermented sausages was obtained by using a combination of traditional and molecular culture-dependent and culture-independent methods, and, with a few exceptions, contributions to the ecological studies of fermented sausages by molecular methods have been available since 2000 (Table 14.2). The picture of fermented sausage microbiota observed after the application of molecular culture-dependent and culture-independent methods correlates with that obtained using biochemical identification; the predominance of *Lact. sakei*, *Lact. curvatus*, and *S. xylosus* emerges. The isolation frequency of *Lact. plantarum* was not as high as that of the other two lactobacilli, although it has also been identified as another important species involved in sausage fermentation. Although their numbers were significantly lower, other *Lactobacillus* species were also identified by traditional methods, such as *Lactobacillus bavaricus* (Hugas et al. 1993; Coppola

**Table 14.2.** LAB and CNC species isolated from traditional fermented sausages.

	Identification method	Source	References
<i>Lact. sakei/curvatus/plantarum/bavaricus</i>	Traditional	Ferm sausages (Spain)	Hugas et al. (1993)
<i>Lact. sakei/curvatus/plantarum; Carnobacterium</i> <i>sp.; Enterococcus sp.; S. saprophyticus/</i> <i>epidermidis</i>	Traditional	Salami (Greece)	Samelis et al. (1998)
<i>Lact. sakei/curvatus; Pediococcus sp.</i>	Traditional	<i>Chorizo</i> (Spain)	Santos et al. (1998)
<i>Lact. sakei/curvatus/bavaricus; S. xylosus/</i> <i>saprophyticus</i>	Traditional	Naples-type salami (Italy)	Coppola et al. (2000)
<i>Lact. sakei/curvatus/plantarum/casei/alimentarius/</i> <i>brevis</i>	PCR-TGGE	Ferm sausages (Italy)	Cocolin et al. (2000)
<i>Lact. sakei/plantarum/curvatus; Leuc. carnosum/</i> <i>gelidum/pseudomesenteroides; Ped. pentosaceus</i>	Traditional	<i>Salsiccia</i> and <i>Soppressata</i> (Italy)	Parente et al. (2001)
<i>Lact. sakei/curvatus/plantarum/paracasei</i>	RAPD-PCR	<i>Soppressata</i> (Italy)	Andriguetto et al. (2001)
<i>Lact. sakei/curvatus/plantarum; S. xylosus</i>	PCR-DGGE	Salami Friulano (Italy)	Cocolin et al. (2001)
<i>Lact. sakei/curvatus/plantarum/buchneri/</i> <i>paracasei; E. faecium; Pediococcus sp.;</i> <i>Leuconostoc sp.</i>	Traditional	Ferm sausages (Greece)	Papamanoli et al. (2003)
<i>Lact. sakei/curvatus/plantarum; E. faecium;</i> <i>S. xylosus/carnosus/epidermidis</i>	S-s PCR	<i>Fuet</i> and <i>Chorizo</i> (Spain)	Aymerich et al. (2003)
<i>Lact. sakei/curvatus/casei; Leuc. mesenteroides;</i> <i>E. casseliflavus; L. lactis; S. xylosus</i>	PCR-DGGE	Fresh sausages (Italy)	Cocolin et al. (2004b)
<i>Lact. sakei/curvatus/plantarum/brevis/</i> <i>paraplantarum; L. lactis; E. pseudoavium;</i> <i>Leuc. citreum/mesenteroides; W. hellenica</i>	PCR-DGGE; RAPD-PCR	Salami Friulano (Italy)	Comi et al. (2005)
<i>Lact. sakei/curvatus/plantarum/casei/</i> <i>paraplantarum; L. lactis; Leuc. citreum/</i> <i>mesenteroides; E. faecium/pseudoavium;</i> <i>Weissella sp.</i>	PCR-DGGE; RAPD-PCR; 16S rDNA seq	Ferm sausages (Greece, Hungary, Italy)	Rantsiou et al. (2005a)
<i>Lact. sakei/curvatus/paracasei; L. garviae;</i> <i>S. xylosus/sciuri/equorum/pulvereri</i>	PCR-DGGE; 16S rDNA seq	Salami Friulano (Italy)	Rantsiou et al. (2005b)
<i>Lact. sakei/curvatus/plantarum; S. saprophyticus/</i> <i>equorum; Enterococcus</i>	PCR-DGGE; 16S rDNA seq	Ferm sausages (Argentina)	Fontana et al. (2005)
<i>Lact. sakei/plantarum/paracasei/pentosus/brevis/</i> <i>rhamnosus; L. lactis; E. faecium; Leuconostoc</i> <i>sp.; S. xylosus/saprophyticus/simulans/</i> <i>gallinarum/cohnii</i>	Traditional	Ferm sausages (Greece)	Drosinos et al. (2005, 2007)
<i>Lact. sakei/curvatus; Leuc. mesenteroides</i>	S-s PCR; RAPD-PCR; plasmid profile	<i>Chorizo, fuet</i> , and <i>Salchichón</i> (Spain)	Aymerich et al. (2006)
<i>Lact. curvatus/plantarum/brevis; Ped.</i> <i>pentosaceus/acidilactici; Leuc. mesenteroides</i>	RAPD-PCR	<i>Salchichón</i> and <i>Chorizo</i> (Spain)	Benito et al. (2007)
<i>Lact. sakei/curvatus; S. xylosus/succinus/equorum</i>	PCR-DGGE; 16S rDNA seq	<i>Soppressata</i> (Italy)	Villani et al. (2007)
<i>Lact. sakei/curvatus/plantarum; L. lactis;</i> <i>S. xylosus/equorum/saprophyticus</i>	ARDRA-PCR; PCR-DGGE	<i>Ciauscolo</i> salami (Italy)	Aquilanti et al. (2007)
<i>Lact. sakei/plantarum/brevis; Leuc. carnosum/</i> <i>mesenteroides; Ped. pentosaceus</i>	ARDRA-PCR; RAPD-PCR	Ferm sausages (Italy)	Bonomo et al. (2008)
<i>Lact. sakei/curvatus/plantarum/pentosus/</i> <i>rhamnosus/brevis/paracasei/alimentarius/</i> <i>fermentum/bavaricus; L. lactis; E. faecium;</i> <i>Ped. pentosaceus/acidilactici; Leuc.</i> <i>mesenteroides; S. xylosus/simulans/carnosus/</i> <i>saprophyticus/capitis/warneri</i>	API system; APILAB Plus	Ferm sausages (Sout-east Europe)	Kozačinski et al. (2008)

*Lact* = *Lactococcus*; *L* = *Lactococcus*; *C* = *Carnobacterium*; *Leuc* = *Leuconostoc*; *W* = *Weissella*; *Ped* = *Pediococcus*; *E* = *Enterococcus*; *S* = *Staphylococcus*.

et al. 2000; Kozačinski et al. 2008); *Lactobacillus casei* and *Lactobacillus paracasei* (Cocolin et al. 2000, 2004b; Andrighetto et al. 2001; Papamanoli et al. 2003; Drosinos et al. 2005; Rantsiou et al. 2005a, 2005b; Kozačinski et al. 2008); *Lactobacillus brevis* (Cocolin et al. 2000; Drosinos et al. 2005; Comi et al. 2005; Benito et al. 2007; Bonomo et al. 2008; Kozačinski et al. 2008); *Lactobacillus plantarum* (Comi et al. 2005; Rantsiou et al. 2005a); *Lact. alimentarius* (Cocolin et al. 2000; Kozačinski et al. 2008); *Lactobacillus rhamnosus* (Drosinos et al. 2007; Kozačinski et al. 2008); *Lactobacillus buchneri* (Papamanoli et al. 2003); *Lact. pentosus* and *Lactobacillus fermentum* (Drosinos et al. 2005; Kozačinski et al. 2008). In the presented studies (Table 14.2), *Leuc. mesenteroides* was the species most frequently isolated, in the genus *Leuconostoc*, from different fermented sausages, while representatives of the genus *Carnobacterium* and *Weissella* were sporadically isolated, which underlines possible pitfalls in their identification or simply their low incidence in this ecological niche. Moreover, these LAB genera are considered undesirable contaminants due to their heterofermentative metabolism. Significant differences in the presence and persistence of enterococci were reported from fermented sausages (Samelis et al. 1998; Papamanoli et al. 2003; Aymerich et al. 2003; Cocolin et al. 2004b; Comi et al. 2005; Fontana et al. 2005; Rantsiou et al. 2005a; Kozačinski et al. 2008). In spite of the controversial presence of *Enterococcus* in foods, meat enterococci, especially *Enterococcus faecium*, have a much lower pathogenicity potential than clinical strains (see Chapter 19), and indeed some of them are used as starter cultures due to their competitiveness in the meat environment and their contribution to final flavor and safety (Franz et al. 2003; Hugas et al. 2003). As regard as pediococci, even when these LAB are among the most common starter culture used in meat fermentation in the United States, in Europe they are not frequently isolated. However, *Pediococcus pentosaceus* and *Pediococcus acidilactici* were often isolated during dry-sausages fermentation with either classical or molecular methods (Santos et al. 1998; Parente et al. 2001; Benito et al. 2007; Bonomo et al. 2008;

Kozačinski et al. 2008). With respect to the CNC microbiota, the species most commonly isolated independent of the country and the technology used is *S. xylosus* (Table 14.2). Other *Staphylococcus* species frequently isolated from meat fermentation include *Staphylococcus saprophyticus* and *Staphylococcus equorum*, which are isolated from different geographical regions (Argentina, Italy, Spain, and Southeast Europe) using both culture-dependent and -independent methods.

The PCR-DGGE technique has been shown to be the most suitable method for microbiota profiling in fermented sausages. When this method was applied to the study of bacterial dynamics in traditional Friulano salami, *Lact. sakei*, *Lact. curvatus*, and *Lact. plantarum* were shown to be the main species strongly present after analyzing both DNA and RNA samples, while *S. xylosus* was found representing CNC (Cocolin et al. 2001; Comi et al. 2005; Rantsiou et al. 2005a, 2005b). When DGGE analysis was applied to Argentinean fermented sausages, *Lact. sakei* and *Lact. plantarum* were found to be mainly responsible for the strong LAB activity during 14 days of fermentation, while *S. equorum* and *S. saprophyticus* dominated among CNC population (Fontana et al. 2005). Recently, Villani et al. (2007), applying both culture-dependent and culture-independent approaches to the study of *Soppressata* of Vallo di Diano, identified *Lact. sakei*, *Lact. curvatus*, *S. xylosus*, *S. equorum*, and *Staphylococcus succinus* as the dominant species occurring during fermentation. Considering the abundant data available on fermented sausages microbiota, it can be concluded that a strong correlation exists between culture-dependent and culture-independent methods of identification, highlighting the fact that *Lact. sakei*, *Lact. curvatus*, and *S. xylosus* are the species best adapted to fermented sausage ecosystems, thereby dominating the present microbiota. However, the differences in organoleptic profiles and sensory characteristics observed in traditional products coming from different countries and produced using different technologies and raw materials can be mainly ascribed to the microbiota involved. Since the main species found in the sausages studied so far were the same among the different products, it

can be suggested that plant-specific biotypes develop within the species (Rantsiou et al. 2005a). This microbiota, usually referred to as “house flora” and resulting from the selective action of production conditions, will predominate during fermentation and characterize the final product of the specific plant. These well-adapted biotypes are promising strains for the development of autochthonous starter cultures that will allow sausages to be produced with both high sanitary and sensory quality (Casaburi et al. 2007; Talon et al. 2008).

#### 14.4.3. Cooked Ready-to-Eat Meat Products

Spoilage of thermally processed VP and MAP meats usually results from the rapid increase in the number of LAB which are able to proliferate during refrigerated storage and are psychrotrophic, microaero-

philic, and resistant to nitrite and salt. Other bacterial groups such as Enterobacteriaceae, yeasts and pathogens (*S. aureus* and *L. monocytogenes*) are unable to prevail in this meat ecological niche, their growth being generally controlled by factors such as nitrite, low storage, temperatures, and competitive LAB populations (Franz and von Holy 1996). As in raw meat ecosystems, LAB are present in ready-to-eat meat and poultry products as a consequence of mishandling and incorrect storage conditions after processing. Studies on the diversity of LAB involved in the spoilage of VP and MAP processed meats have shown that *Leuconostoc* as well as *Weissella* and *Carnobacterium* dominates the spoilage bacterial associations (Table 14.3). In an early study, Franz and von Holy (1996) showed that LAB isolated from VP Vienna sausages ranged from 53% to 75% of the total isolated strains depending on pasteurization conditions; this population accounted

**Table 14.3.** Major LAB species isolated from cooked ready-to-eat meat and poultry products.

	Identification method	Source	References
Homo and heterofermentative lactobacilli; <i>Leuconostoc</i> ;	Traditional	Pasteurized VP Vienna sausages; 8°C (South Africa)	Franz and von Holy (1996)
<i>Pediococcus</i> ; <i>Bacillus</i>	Traditional	Cooked whole-meat products, emulsion-type sausages; 4°C (Greece)	Samelis et al. (2000)
<i>Lact. sakei/curvatus</i> ; <i>Leuc. mesenteroides/carnosum</i> ;	Ss-PCR	Cooked MAP poultry meat; 3.5°C (Canada)	Barakat et al. (2000)
<i>L. citreum</i> ; <i>W. viridescens</i>	RFLP; WCPP; 16S rDNA seq; DNA–DNA hybridization	Marinated MAP broiler meat strips; 6°C (Finland)	Björkroth et al. (2000)
<i>L. raffinolactis/garviae/lactis</i> ;	PCR-DGGE	Cooked ham, Vienna sausage, roasted pork (Japan)	Takahashi et al. (2004)
<i>C. divergens/piscicola</i> ;	RFLP; 16S rDNA seq; DNA–DNA hybridization	Marinated MAP broiler meat; 6°C (Finland)	Koort et al. (2005)
<i>E. faecalis</i>	RFLP	<i>Morcilla de Burgos</i> ; 4°C (Spain)	Santos et al. (2005)
<i>Leuc. gasicomitatum</i>	CFA–Gas chromat; RFLP	Cooked ham; 4°C (Greece)	Samelis et al. (2006)
<i>Leuc. carnosum</i> ; <i>W. hellenica</i>	S-s PCR; ARDRA	Cooked ham, chopped turkey, <i>morcilla, fiambre de magro adobado</i> ; 4°C (Spain)	Chenoll et al. (2007)
<i>Lact. oligofermentans</i>	Rep-PCR; PCR-DGGE	MAP artisan-type cooked ham; 4 to 26°C (Belgium)	Vasilopoulos et al. (2008)
<i>Leuc. carnosum/mesenteroides</i> ;			
<i>W. viridescens/confuse</i> ;			
<i>Lact. fructosus</i>			
<i>Leuc. plantarum/sakei/curvatus</i> ;			
<i>Leuc. mesenteroides</i> ; <i>C. mobile</i> ;			
<i>divergens/maltaromaticum</i>			
<i>Leuc. carnosum</i> ; <i>C. divergens</i> ;			
<i>E. faecalis</i>			

*Lact* = *Lactobacillus*; *L* = *Lactococcus*; *C* = *Carnobacterium*; *Leuc* = *Leuconostoc*; *W* = *Weissella*; CFA-gas chromat = cellular fatty acids-gas chromatography.



mainly for *Leuconostoc* and homofermentative lactobacilli. When the selective effect of the product type and the packaging conditions on LAB diversity was evaluated, cured whole-meat products (ham, pork loin, bacon, and turkey breast fillets) showed *Leuc. mesenteroides*, *Leuc. carnosum*, and *Lact. sakei* as dominant LAB (Samelis et al. 2000). Microbial associations of emulsion-type sausages (pariza, mortadella, and frankfurters) were more diverse, also involving *Lact. curvatus*, *Leuc. citreum*, *Leuc. gelidum*, and *Weissella viridescens*. When phenotypic and PCR-based combined methods were used to characterize the dominant microbiota of MAP cooked poultry meat injected with commercial brine, strains of *Lactococcus raffinolactis*, followed by *C. divergens* and *Carnobacterium piscicola*, were predominately identified, probably as post-cooking contamination (Barakat et al. 2000). On the other hand, when LAB population was investigated in “morcilla de Burgos,” a typical Spanish blood cooked sausage, by means of phenotypic characteristics and ribotyping, *W. viridescens* was the main species detected, followed by *Leuc. mesenteroides*, *Leuc. carnosum*, and *Weissella confusa* (Santos et al. 2005). By a polyphasic taxonomic approach, Samelis et al. (2006) verified that the major group of atypical *Leuconostoc*-like bacteria that dominated the spoilage microbiota of refrigerated whole cooked hams produced in Greece was *Leuc. carnosum*, whose presence is associated with raw pork chilled meat used for making hams. The use of rDNA-based molecular techniques provided a reliable description of LAB composition in different VP cooked meat products, pointing to *Leuc. mesenteroides* as the main species present and the one responsible for bloating spoilage in these products, and *Lact. sakei*, *Lact. plantarum*, and *Lact. curvatus* being found in decreasing order of abundance (Chenoll et al. 2007). Recently, the combination of culture-dependent and culture-independent approaches applied to Belgian MAP artisan-type cooked ham stored at different temperatures (4–26°C) revealed that bacterial population was dominated by *Leuc. carnosum* and *C. divergens* at all storage conditions (Vasilopoulos et al. 2008). Enterococci, mainly *Ent. faecalis*, became detectable at higher temperatures, while *Lact. sakei*

and *L. lactis* were present at the very early stages of spoilage, outcompeted by other members of meat-borne microbiota. Similarly, PCR-DGGE applied on spoiled meat products (Vienna sausages, roast pork, and cooked ham) and processing facilities allowed *Leuc. carnosum* and *W. hellenica* to be quickly identified as the LAB responsible for product contamination (Takahashi et al. 2004). In addition, novel species such as *Leuconostoc gasicomitatum*, *Lactobacillus oligofermentans*, and *Enterococcus hermanniensis* have been associated with the spoilage of MAP marinated broiler meat strips and poultry products (Björkroth et al. 2000; Koort et al. 2004, 2005). Under MAP conditions, *B. thermosphacta* was also found, and even when its presence appeared to be stimulated by increased O<sub>2</sub> concentrations (Samelis et al. 2000; Cayré et al. 2005; Vasilopoulos et al. 2008), it became outcompeted by LAB, mainly through bacteriocin production (Castellano and Vignolo 2006). In ready-to-eat meat and poultry ecosystems, LAB presence is linked to the production of acids, off-flavors, and color-deteriorating compounds, often leading to product rejection. However, the selection of competitive naturally growing bacteriocinogenic LAB strains could be an effective way of controlling these LAB spoilers that cause undesirable organoleptic changes in VP and MAP meat.

## 14.5. Conclusions

The biodiversity that exists in traditional fermented foods is a significant source of biotechnological products and processes innovation. Knowledge of LAB diversity present in meat ecosystems as well as the structure and function of microbial consortiums in which they are involved allow their use for the design of autochthonous functional cultures. The microbial diversity described for the different meat ecosystems reveals a high intraspecies polymorphism since a consistent dominance of LAB species was reported. The speciation diversity arising from the selective environmental pressures (NaCl, nitrites, high or low temperatures, oxygen concentration) that are prevalent in each meat ecosystem may be ascribed to different functionalities of LAB strains.

To avoid the loss of traditional fermentation uniqueness and ensure food safety, the development of a new generation of LAB cultures offering health, marketing, and technological advantages relies on metagenomics as a tool for providing effective strategies to improve the functionality and safety of foods. Global approaches based on proteomics and transcriptomics are in progress and will allow for a better understanding of LAB interactions with the meat substrate.

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## Chapter 15

# New Trends in Cereal-based Products Using Lactic Acid Bacteria

Graciela Font de Valdez, Carla L. Gerez, María Inés Torino, and Graciela Rollán

*The global importance of cereals to human diet and, moreover, to the history of civilization, in particular agriculture, cannot be overstated. Nutritionally, cereals are an important source of dietary proteins, carbohydrates, vitamins E and B-complex, iron, trace minerals, and fiber. Major cereal crops produced worldwide include wheat, rice, maize, rye, and barley, their main application being in bread manufacture (20–106 kg per capita annually). Only wheat and rye are suitable for the elaboration of leavened bread because of the presence of sufficient amounts (8%–14%) of the complex protein gluten. Other common uses of cereals are in the preparation of fermented traditional products, such as Ogi (maize, millet, or sorghum), Kenkey (maize), and Gari (cassava root), as well as drinks. In these fermented cereal-based products, different species of lactic acid bacteria (LAB) belonging to the genus Lactobacillus, Leuconostoc, Weissella, Pediococcus, Lactococcus, Enterococcus, and Streptococcus are involved. Lactic fermentation improves food quality not only by enhancing flavor development and increasing the nutritional value of food but also by extending their shelf life and by removing toxic and anti-nutritional factors. Cereals have become suitable substrates for innovation in the market for functional foods containing prebiotics and/or probiotic LAB strains.*

### 15.1. Introduction

In almost every country and region, cereals provide the staple food. Cereals have been, and still are,

the most important food crop. Their cultivation dates back to 7000 B.C. for wheat and barley, 4500 B.C. for rice and maize, 4000 B.C. for millet and sorghum, 400 B.C. for rye, and 100 B.C. for oats. In developed countries, up to 70% of the cereal harvest is used as animal feed, while in developing countries cereal is mainly used for human nutrition (Hammes et al. 2005). Cereal grains contain macronutrients (proteins, fats, and carbohydrates) required by humans for growth and maintenance. They also provide important minerals, vitamins, and other micronutrients essential for optimal health.

Food fermentation is one of the oldest processing technologies known to humans with the primary purpose of achieving a preservation effect. Since the early civilization, methods for the fermentation of milks, meats, and vegetables have been described, with records dating back to 6000 B.C., and the civilizations of the Fertile Crescent in the Middle East (Fox 1993). Fermented foods prepared from most common types of cereals (such as rice, wheat, corn, or sorghum) are well known in many parts of the world. A range of indigenous cereal-based fermented foods is presented in Table 15.1. These foods are produced using various manufacturing techniques, raw materials, and microorganisms, although only four main fermentation processes exist: alcoholic, lactic, acetic, and alkali. Most of the foods listed in Table 15.1 are produced in Africa and Asia, and a number of them use cereals in combination with legumes, thus improving the overall protein quality of the fermented product. Cereals are

**Table 15.1.** Examples of cereal-based fermented foods.

Product	Country	Microorganisms	Substrate
Boza	Turkey, Albania, Bulgaria	<i>Lactobacillus</i> , <i>S. cerevisiae</i> , <i>Leuconostoc</i>	Wheat, millet, maize, and other cereals
Bread	International	<i>S. cerevisiae</i> , other yeasts, lactic acid bacteria	Wheat, rye, other grains
Idli	Southern India	Lactic acid bacteria ( <i>Leuc. mesenteroides</i> , <i>Enterococcus faecalis</i> ) <i>Torulopsis</i> , <i>Candida</i>	Rice and black gram dhal
Kenkey	Ghana	<i>Lact. fermentum/reuteri</i> , yeast, molds	Maize
Mahewu	South Africa	Lactic acid bacteria	Maize
Nan	India	Lactic acid bacteria, <i>S. cerevisiae</i>	White wheat flour
Ogi	Nigeria, West Africa	Lactic acid bacteria, <i>Cephalosporium</i> , <i>Fusarium</i> , <i>Aspergillus</i> , <i>Penicillium</i> spp., <i>S. cerevisiae</i> , <i>Candida mycoderma</i>	Maize
Pozol	South-eastern Mexico	<i>L. lactis</i> , <i>Lact. casei/plantarum/alimentarium</i>	Maize
Uji	Kenya, Uganda	<i>Lact. plantarum</i> , <i>Leuc. mesenteroides</i>	Maize, sorghum, millet

deficient in lysine but are rich in cysteine and methionine. Legumes, on the other hand, are rich in lysine but deficient in sulfur-containing amino acids.

Fermented cereal-based food products produced in African countries can be classified either on the basis of the raw cereal ingredients used in their preparation or on the basis of texture. The first classification includes: (1) wheat-based foods (e.g., bouza, kishk); (2) rice-based foods (e.g., busa); (3) maize-based foods (e.g., ogi, bread, kenkey); (4) millet-based foods (e.g., kunuzaki); and (5) sorghum-based foods (e.g., pito, ogi, bogobe, kiswa, burukutu, kiswa, injera). Regarding texture, the classifications are: (1) liquid (gruel) (e.g., ogi, mahewu, burukutu, pito, uji); (2) solid (dough) and dumplings (e.g., kenkey, agidi); and (3) dry (bread) (e.g., kiswa, injera) (Blandino et al. 2003).

This chapter provides an overview of the role and functionality of lactic acid bacteria (LAB) in cereal-based products and sourdough fermentation.

## 15.2. Sourdough Bread

For several thousand years, bread has been one of the major constituents of the human diet. Sourdough is a mixture of flour (e.g., wheat flour) and water spontaneously fermented by LAB and yeasts related to traditional and original foods (Hammes et al. 2005). Sourdough is an intermediate product for

dough and bread preparation that contains metabolically active microorganisms. Interest in using sourdough for bread production has increased greatly during the last few years due to new trends in foods with “history” (Hansen and Schieberle 2005). Thus, the constant demand for natural foods in European countries has increased the demand for sourdough breads. Production of traditional rye breads in Nordic, Central, and Eastern European countries includes addition of sourdough to the bread dough and therefore to wheat bread production in the Mediterranean area (Italy, Spain, Morocco, and Egypt). In the United States, the tradition of sourdough fermentation is known from the San Francisco Bay Area (Spicher and Stephan 1999; Hansen and Schieberle 2005).

German sourdoughs are usually rye-based mainly due to the difficulties of growing wheat under the German harsh climate conditions, especially in the northern and eastern regions of the country. Furthermore, dough acidification is required for rye baking to inhibit flour  $\alpha$ -amylase to prevent excessive starch degradation during baking and to obtain an acceptable bread volume (Brandt 2007). In addition, acidification (biological or chemical) leads to a substantial degradation of glutelins probably by the activation of endogenous proteolytic enzymes in rye flour (Thiele et al. 2004). Due to the artisan and region-dependent handling of sour-



doughs, they are a huge source of LAB and yeast species diversity.

### 15.2.1. Biodiversity

The type of bacterial flora developed in each fermented food depends on water activity, pH, salt concentration, incubation temperature, and composition of the food matrix. The concerted hydrolytic activities of the grain and microorganisms (LAB and yeasts) are the origin of all cereal fermentations and are best represented by the traditional sourdough fermentation. On the basis of the technology used in sourdough production, three types of sourdoughs have been identified (Böcker et al. 1995). Type I sourdoughs are produced with traditional techniques and characterized by continuous refreshments to keep the microorganisms in an active state. Type II sourdoughs, often used as dough-souring supplements during bread preparation, are characterized by long fermentation periods (from 2 to 5 days) and fermentation temperature sometimes higher than 30°C to speed up the process (Böcker et al. 1995; Hammes and Gänzle 1998). Type III sourdoughs are dried preparations containing LAB that are resistant to the drying process (Hammes and Gänzle 1998).

Sourdough LAB generally belong to the genus *Lactobacillus*, *Leuconostoc*, *Pediococcus*, or *Weissella*, the first one exhibiting the greatest diversity. More than half of the species (approximately 100) of the *Lactobacillus* genus occur in sourdoughs or related cereal fermentations (Hammes and Hertel 2003; De Vuyst and Neysens 2005; Ehrmann and Vogel 2005). Table 15.2 shows LAB diversity isolated from sourdoughs from different regions and the phenotypic and/or molecular methods applied in their identification. Interestingly, 15 lactobacilli species known to occur in sourdough are also known to live in human and animal intestines, for example, *Lactobacillus reuteri* and *Lactobacillus acidophilus* (Hammes and Gänzle 1998). *Lactobacillus alimentarius* strains, which probably belong to *Lactobacillus paralimentarius*, were first isolated from Japanese sourdough (Cai et al. 1999). *Lactobacillus brevis* and *Lactobacillus plantarum* have been generally

associated with *Lactobacillus fermentum* in Russian sourdoughs (Kazanskaya et al. 1983), while *Lact. fermentum* dominates Swedish sourdoughs (Spicher and Lönner 1985) and German type II sourdoughs (Meroth et al. 2004). Furthermore, Gobbetti et al. (1994b) reported that *Lact. acidophilus* is common in Umbrian (Italian region) sourdoughs, although it has been rarely isolated from other sourdoughs (Salovaara and Katunpää 1984; Spicher 1984; Infantes and Tourneur 1991).

In the last years, several new lactobacilli species have been discovered in traditional sourdoughs by using polyphasic approaches (Table 15.2). Four of the novel taxa, *Lactobacillus acidifarinae*, *Lactobacillus hammesii*, *Lactobacillus spicheri*, and *Lactobacillus zymae*, are members of the *Lactobacillus buchneri* species group; *Lactobacillus mindensis* and *Lactobacillus nantensis* enrich the species diversity of the *Lact. alimentarius* species group; and *Lactobacillus rossiae* belongs to the *Lact. reuteri* species group (De Vuyst and Vancanneyt 2007). In addition, a ubiquitous microbiota involving pediococci and heterofermentative lactobacilli were found in homemade sourdoughs from the mountain area of north-west Argentina (Gerez et al. 2006). From this geographical region, polyphasic analysis applied to fermented wheat flour allowed the identification of a novel species within the genus *Pediococcus*; *Pediococcus argentinicus* sp. nov. (De Bruyne et al. 2008).

## 15.3. Functionality of LAB in Sourdough Fermentations

Following the identification and classification of LAB from cereal fermentations, basic and applied sciences now face the challenge of identifying functional characters of these bacteria to completely exploit their microbial metabolic potential for the production of baked goods (Vogel et al. 2002). The deliberate exploitation of desired metabolic activities would be possible if the contribution of individual metabolic behavior to bread quality is well defined (Ehrmann and Vogel 2005).

The metabolic activities of LAB during sourdough fermentation improve dough properties,

**Table 15.2.** Lactic acid bacteria from sourdoughs of different origin.

LAB	Product (identification method)	Country	Reference
<i>Ped. argentinus</i>	Wheat sourdoughs (polyphasic)	Argentina	De Bruyne et al. (2008)
<i>Lact. zymae/pontis/Acidifarinae/plantarum</i>	Wheat sourdoughs (polyphasic)	Belgium	Vancanneyt et al. (2005)
<i>sanfranciscensis/paralimentarius/spicheri</i>			Scheirlinck et al. (2007, 2009)
<i>Lact. reuteri/panis/amylovorus</i>	Rye sourdough (phenotypical)	Denmark	Rosenquist and Hansen (2000)
<i>Lact. plantarum/casei/delbrueckii</i> subsp. <i>delbrueckii/acidophilus/brevis/curvatus</i> ; <i>Ped. pentosaceus</i>	Wheat bread (phenotypical)	France	Infantes and Tourneur (1991)
<i>Lact. hammesii</i>	Wheat sourdoughs (polyphasic)		Valcheva et al. (2005)
<i>Lact. nantensis</i>	Wheat sourdoughs (polyphasic)		Valcheva et al. (2006)
<i>Lact. amylovorus/pontis/frumenti/reuteri</i>	Rye sourdough (RAPD-PCR)	Germany	Müller et al. (2000, 2001)
<i>Lact. panis</i>	Rye sourdough (polyphasic)		Wiese et al. (1996)
<i>Lact. spicheri</i>	Rice sourdough (polyphasic)		Meroth et al. (2004)
<i>Lact. pontis/Lact. sanfranciscensis/</i> <i>mindensis/crispatus/pontis/fermentum/</i> <i>reuterifrumenti/johnsonii/panis</i>	Rye bran (PCR-DGGE)		Vögel et al. (1994)
<i>Lact. paralimentarius/anfranciscensis/</i> <i>brevis/Lactobacillus</i> sp.; <i>W. cibaria</i>	Wheat sourdoughs (polyphasic)	Greece	Meroth et al. (2003); Ehrmann et al. (2003)
<i>Lact. sanfranciscensis/fermentum/</i> <i>plantarum; Leuc. mesenteroides</i> ; <i>Pediococcus</i> spp.	Panettone, Brioche (phenotypical)	Italy	De Vuyst et al. (2002)
<i>Lact. alimentarius/ brevis/plantarum/</i> <i>sanfranciscensis/lactis</i> subsp. <i>lactis/</i> <i>acidophilus/fermentum/delbrueckii</i> subsp. <i>bulgaricus</i> ; <i>W. confuse/rosii</i>	Apulian wheat sourdoughs (polyphasic)		Galli et al. (1988)
<i>Lact. sanfranciscensis/acidophilus/</i> <i>plantarum/farciminis</i>	Umbrian wheat sourdoughs (phenotypical)		Corsetti et al. (2001, 2005)
<i>Lact. brevis/plantarum</i>	Wheat sourdough (phenotypical)	Spain	Gobbetti et al. (1994b)
<i>Lact. siliginis</i>	Wheat sourdough (polyphasic)	South Korea	Barber et al. (1983)
<i>Lact. sanfranciscensis</i>	S. Francisco sourdough, French bread (phenotypical)	United States	Aslam et al. (2006)
			Kline and Sugihara (1971)

bread texture. and flavor; retard the staling process of bread; and prevent bread from mold and bacterial spoilage (Hansen and Schieberle 2005; Arendt et al. 2007; Gerez et al. 2009). Also, LAB may contribute to the healthiness of cereals in different ways, such as improving the texture and palatability of whole-grain, fiber-rich, or gluten-free products; hydrolyzing allergenic compounds (Rollán et al. 2005; Gobbetti et al. 2007, Gerez et al. 2008b); stabilizing or increasing levels of bioactive compounds; and improving mineral bioavailability (De Angelis et al. 2003; Katina et al. 2005; Arendt et al. 2007).

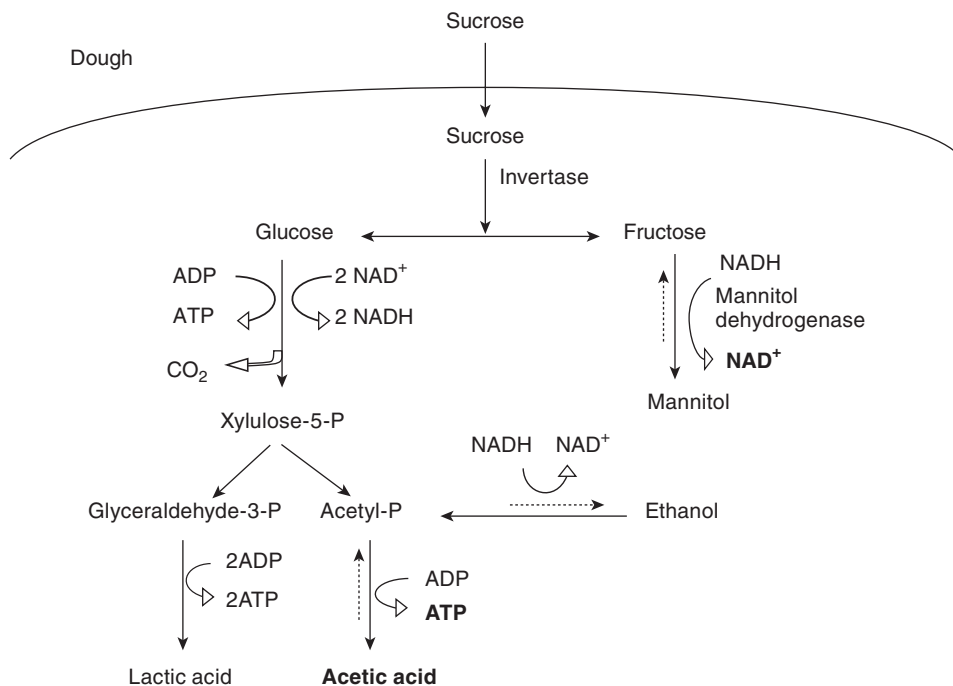
### 15.3.1. Carbohydrate Metabolism during Sourdough Fermentation

In dough fermentation, carbohydrate metabolism varies, depending on the LAB species and strains involved, type of sugars, presence of yeasts, and processing conditions (Hammes and Gänzle 1998; Martínez-Anaya 2003). The soluble carbohydrate concentration of wheat flour is less than 1% and varies with the type of flour and with the action of  $\alpha$ - and  $\beta$ -amylases on the damaged starch granules (Gobbetti et al. 1994a).

Carbohydrate metabolism and physiological aspects of *Lactobacillus sanfranciscensis* and other sourdough lactobacilli in sourdough were reviewed by Hammes et al. (1996), Gobbetti et al. (2005), and Gänzle et al. (2007). *Lact. sanfranciscensis* strains isolated from Italian traditional sourdoughs only use glucose and maltose (Corsetti et al. 2001); however, a broad range of sugars such as sucrose, raffinose, galactose, melibiose, ribose, and fructose are fermented by *Lact. sanfranciscensis* strains isolated from other sourdoughs (Hammes and Gänzle 1998; Tieking et al. 2003).

*Lact. reuteri* CRL1100, a strain isolated from homemade fermented dough, showed a variable profile of carbohydrate fermentation (Ginés et al. 1997). This microorganism metabolizes glucose and galactose but not fructose and cellobiose. Among disaccharides, sucrose and maltose are the best substrates. The consumption of maltose and the release

of glucose from sugar hydrolysis may have favorable impact on maltose-negative yeasts for growing in sourdoughs (Sugihara et al. 1970; Gobbetti et al. 1994a), especially during wheat sourdough fermentation where the concentration of soluble carbohydrates is less than 1% (Ginés et al. 1997). In general, sourdough LAB are unable to ferment sucrose, a disaccharide whose metabolism by heterofermentative lactobacilli in sourdough appears to be attributable to glycosyl-transferases rather than to invertase activities (Tieking et al. 2003; Gänzle et al. 2007). However, Cuezco de Ginés et al. (2000) purified and characterized an invertase ( $\beta$ -fructofuranosidase) enzyme from *Lact. reuteri* CRL1100. During wheat dough fermentation, this strain metabolizes sucrose with a concomitant release of fructose, which is used mainly as an electron acceptor for producing mannitol (Gerez et al. 2008a), changing the metabolism from the ethanol to the acetate route (Fig. 15.1)



**Figure 15.1.** Fermentation of sucrose by *Lact. reuteri* CRL1100, a lactic acid bacterium isolated from homemade sourdough.

(Stolz et al. 1995; Erten 1998; Korakli and Vogel 2003). The reduction of fructose to mannitol allows the regeneration of  $\text{NAD}^+$  and the gain of ATP by the cells through the acetate kinase reaction converting acetyl phosphate into acetate instead of ethanol. The use of fructose as an electron acceptor was also reported for strains of *Leuconostoc mesenteroides* (Erten 1998), *Lact. sanfranciscensis* (Korakli and Vogel 2003), and *Lact. fermentum* (Vrancken et al. 2008). The invertase activity of *Lact. reuteri* CRL1100 increases soluble sugars (Gines et al. 1997, Cuezco de Ginés et al. 2000) and regenerates  $\text{NAD}^+$  (Gerez et al. 2008a) in the sourdough ecosystem, which has positive impact on this ecological system.

#### 15.3.2. Exopolysaccharide Production by Sourdough LAB

Sourdough LAB have been shown to produce exopolysaccharides (EPS): glucans (reuteran, dextran, or mutans) as well as fructans (levan or inulin). The molecular and applied aspects of EPS formation by sourdough LAB were recently reviewed by Tieking and Gänzle (2005) and Gänzle et al. (2007). EPS are natural biopolymers, which play an important role in the rheology, texture, and mouthfeel of fermented milks, nonfat cheeses, and more recently, sourdough or bakery products (Decock and Capelle 2005). *In situ* EPS formation by lactobacilli during sourdough fermentation may replace hydrocolloids currently used as texturizing, anti-staling, or prebiotic additives in bread production (Tieking et al. 2003). The *in situ* production of these compounds is preferred to the use of additives in the elaboration of “100% natural” fermented foods. The potential probiotic effects of EPS and their technological properties, in addition to the Generally Recognized as Safe character of LAB, make these EPS-producing microorganisms ideal to be included in new functional starter cultures or functional foods. The great biodiversity of these biopolymers in terms of yield, monomeric composition, molecular mass, and spatial structure confers them diverse functionalities.

### 15.4. Cereal Protein Modification during Sourdough Fermentation

Gluten proteins (gliadins and glutenins) are responsible for the unique ability of wheat to be transformed into bread; they are responsible for the rheological properties of dough and the texture of breads, contributing to gas retention during dough fermentation (Wieser 2007). The main proteolytic activity of this process was attributed to endogenous flour enzymes, such as aminopeptidases, carboxypeptidases, and endopeptidases (Spicher and Nierle 1988). The degree of protein degradation in wheat sourdough is usually similar to that observed in chemically acidified doughs (Loponen et al. 2004; Thiele et al. 2004), although lower peptide and amino acid levels were found in chemically acidified doughs (Di Cagno et al. 2004). During dough fermentation, the proteolysis carried out by LAB releases small peptides and free amino acids, which are important for rapid microbial growth and acidification and as precursors for flavor development of the leavened baked products (Rollán and Font de Valdez 2001; Thiele et al. 2002). Gerez et al. (2006) reported that 13 (nine lactobacilli and four pediococci) out of 42 LAB strains were able to use gluten as a nitrogen source. Pediococci displayed similar proteolytic activity to lactobacilli, providing the first evidence of gluten proteolysis by sourdough pediococci strains. The amino acid levels in wheat doughs depend on dough pH, fermentation time, and amino acid consumption by the fermentative microflora (Thiele et al. 2002). An increase (80% and 150%) in basic amino acids (mainly ornithine) and lysine in 6h-old *Pediococcus pentosaceus* cultures grown in gluten-based medium was observed. Ornithine is considered one of the key flavor precursors in wheat bread (Gassenmeier and Schieberle 1995). Gerez et al. (2006) observed an increase in essential amino acids (treonine, valine, lysine, and phenylalanine) in a gluten-based medium fermented by LAB strains. As cereal flours used for human or animal foods are deficient in essential amino acids (Kamel and Stauffer 1993), the inclusion of lysine-releasing pediococci in starter cultures for wheat dough would be relevant from a nutritional standpoint. In addi-

tion, the qualitative and quantitative composition of amino acids in fermented doughs is of interest since they positively affect bread flavor mainly through the Maillard reaction (Collar et al. 1991).

Studies on the proteolytic system of sourdough LAB contribute to the selection of starter cultures that have the ability to eliminate or metabolize toxic peptides involved in celiac disease (CD) (Di Cagno et al. 2002; Rollán et al. 2005; Zotta et al. 2006; Gerez et al. 2008b). CD is an inflammatory disorder of the upper small intestine triggered by the ingestion of wheat, rye, barley, and possibly oat products. The clinical feature of CD is characterized by a flat intestinal mucosa with absence of normal villi, which results in a generalized malabsorption of nutrients. During the last decade, intensive biochemical studies have contributed to substantial progress in understanding the general principles that determine the pathogenesis of CD.

The structural features unique to all CD toxic proteins are sequence domains rich in glutamine (Gln) and proline (Pro) (Sollid 2002). The high Pro content renders these proteins resistant to complete proteolytic digestion by gastrointestinal enzymes (Cunningham and O'Connor 1997; Hausch et al. 2003). Consequently, large Pro- and Gln-rich peptides are accumulated in the small intestine and reach the sub-epithelial lymphatic tissue. Depending on the amino acid sequences, these peptides can induce two different immune responses that result in mucosal destruction and epithelial apoptosis. The current essential therapy of CD is a strict lifelong adherence to gluten-free diet. Dietetic gluten-free foods produced for CD patients underlie the regulations of the Codex Alimentarius Standard for Gluten-Free Foods. The "Draft Revised Codex Standard," edited in March 2006, proposes a maximum level of 20 mg of gluten/kg for naturally gluten-free foods (e.g., based on rice or corn flour) and 200 mg/kg for foods rendered gluten-free (e.g., wheat starch). There is still disagreement about the toxicity of oat avenins. During the last decade, strategies for prevention and treatment of CD have been proposed. They are based on the removal of toxic epitopes by enzymatic degradation, gene engineering, and blocking parts of the immune system (Shan

et al. 2002; Di Cagno et al. 2004; Stepniak et al. 2006). However, any alternative treatment should have a safety profile competitive with gluten-free diet (Wieser and Koehler 2008). On the other hand, gluten-free breads tend to have a flat aroma and stale fast. It has been reported that for wheat bread both of these disadvantages can be overcome by using sourdough (Clarke et al. 2002). Different publications demonstrated that LAB possess great potential in diminishing the CD-inducing effects of gluten. It was shown that selected lactobacilli have the ability to *in vitro* hydrolyze Pro-rich peptides, including the 31–43  $\alpha$ -gliadin and the 57–89  $\alpha$ -gliadin (33-mer peptide) fragments, the latter being the most potent inducer of gut-derived human T cell lines in CD patients (Di Cagno et al. 2002; Rollán et al. 2005). On the other hand, Shan et al. (2005) reported the use of a prolyl-endopeptidase produced by *Flavobacterium meningosepticum* active on the 33-mer peptide, for an oral peptidase supplement therapy for CD patients. Nonetheless, Matysiak-Budnik et al. (2004) reported that the hydrolysis of the 33-mer by prolyl-endopeptidase of *F. meningosepticum* in CD patients was not complete and led to the release of potentially immunogenic peptides, which contacted the immune system after crossing the intestinal mucosa. Other prolyl-endopeptidase from *Aspergillus niger* efficiently degraded T cell stimulatory peptides as well as peptic/tryptic digest of gluten and intact gluten, and this enzyme was proposed as an oral supplement to reduce gluten intake in CD patients (Stepniak et al. 2006). Moreover, the combination of LAB strains and fungal proteases decreased the level of residual gluten under 20 mg/kg in dough, considered an efficient approach for reducing gluten in baked goods (Gobbetti et al. 2007; Rizzello et al. 2007).

Recently, Gerez et al. (2008b) reported no correlation between the individual peptidase activities of LAB strains and their capacity to degrade  $\alpha$ -gliadin-derived fragments. However, some strains were able to hydrolyze (more than 70%) the 31–43 and 62–75  $\alpha$ -gliadin-fragments after 2 h, while the degradation of the 33-mer peptide was only obtained by using mixed cultures with different peptidase profiles after 8 h (pers. comm.). This fact would be



due to the synergic or complementary effect among the enzyme activities. The degradation rate of the 33-mer peptide, the most resistant peptide to LAB hydrolysis, was higher than the value reported by a pool of four LAB strains isolated from sourdough (Di Cagno et al. 2004).

#### 15.4.1. Role of Amino Acids during Sourdough Fermentation

Certain amino acids released by proteolysis during sourdough fermentation have been shown to play different roles. One important metabolic activity of sourdough lactobacilli is the arginine (Arg) catabolism through the Arg deiminase (ADI) pathway. Degradation of this amino acid has significance for bread quality and helps to protect the microorganism in acidic environments (Thiele et al. 2002; Rollán et al. 2003). The ADI pathway comprises three reactions catalyzed by ADI (EC 3.5.3.6.), ornithine carbamoyl-transferase (EC 2.1.3.3.), and carbamate kinase (EC 2.7.2.2.). This pathway leads to the conversion of Arg into ornithine, ammonia, and CO<sub>2</sub>, with the concomitant production of 1 mol of ATP per mol of Arg consumed; this system is the main Arg catabolic pathway in many LAB strains. The arginine metabolism by *Lact. sanfranciscensis* (De Angelis et al. 2002) and *Lactobacillus pontis* (Thiele et al. 2002) has been demonstrated to have impact on bread flavor. *Lact. pontis* is capable of metabolizing Arg forming the aroma precursor ornithine, which is converted into 2-acetyl-1-pyrroline, giving the bread crust its aroma (Schieberle 1990, 1996; Ehrmann and Vogel 2005). Furthermore, the metabolism of Arg into ornithine *via* the ADI pathway contributes to pH-homeostasis and the acid tolerance of lactobacilli because two protons are consumed in the pathway increasing both the intracellular and extracellular pH (Konings 2002). In *Lact. reuteri*, the triggering factor for the ADI pathway would be the depletion in energy source rather than the pH attained by cultures at the stationary phase (Rollán et al. 2003).

Other amino acids derived from the gluten breakdown during LAB fermentation are glutamate (due to glutamine deamidation) and the essential amino

acids treonine, valine, lysine, and phenylalanine (Rollán et al. 2005; Gerez et al. 2006; Vermeulen et al. 2007). Phenylalanine is of special interest since its metabolism leads to the production of phenyllactic acid (PLA) and its 4-hydroxy derivative (OH-PLA), both of which have been described as antifungal compounds (Lavermicocca et al. 2000; Valerio et al. 2004).

### 15.5. Bread Bioconservation by LAB

Bread is one of the most important staple foods in the Western world and is generally viewed as a perishable commodity. Mold growth is the most common microbial spoilage in bakery products, raising serious economic concerns as due to production losses of between 1% and 5% depending on season, type of products (bread or sweet baked goods), ingredients (flour), leavening source (chemical, baker's yeasts, or sourdough), and processing methods. The characteristics, architecture, and cleaning of bakery and packaging products are also determinant factors since spores of airborne molds contaminate baked goods during cooling, slicing, wrapping, and storage (Legan 1993). Spoilage of wheat bread is mainly linked to the presence of *Penicillium* sp., which causes around 90% of bread spoilage, but *Fusarium*, *Cladosporium*, *Rhizopus*, and *Aspergillus* species also occur (Legan and Voysey 1991). On rye bread, *Penicillium roqueforti* is the major contaminant (Lund et al. 1996).

In addition to its economic impact, the presence of mold in bakery goods may be associated with the production of mycotoxins, posing public health risks (Legan 1993). At present, several methodologies are applied to prevent or minimize fungal spoilage of bread and to ensure shelf life and safety of bakery products, for example, modified atmosphere packaging; cold storage; irradiation; pasteurization of packaged bread; and/or addition of preservatives such as propionic, sorbic, and benzoic acids and their salts (Farkas 2001; Guynot et al. 2005), with propionic and sorbic acids used mainly as food preservatives. According to the European Parliament and Council Directive No. 95/2/EC, propionic and sorbic acids may be added to bakery wares in con-

centrations of up to 0.3% and 0.2%, respectively (European Community 1995).

Consumer demands for natural food products have led the bakery industry to lower the quantities of chemical additives used (Membre et al. 2001). However, the reduction of preservatives to sub-inhibitory levels has been shown to stimulate growth of spoilage fungi in some cases (Marin et al. 1999), and/or mycotoxin production (Bullerman 1985; Legan 1993). Demands in the bakery industry for high-quality and minimally processed foods with low concentrations of chemical preservatives renewed the interest toward natural preservation systems. In this regard, considerable resources have been devoted to the biotechnology of LAB as bio-preserver organisms since they have been used for centuries in the food industry and are able to produce different kinds of antimicrobial compounds (Diep and Nes 2002; Cotter et al. 2005; Drider et al. 2006). Some LAB have proven to affect spoilage and mycotoxigenic fungi because of their acidification activity and the antifungal substances they produce. Several LAB antimicrobial low-molecular-weight compounds including organic acids (Cabo et al. 2002; Lind et al. 2007; Gerez et al. 2009), reuterin (Talarico et al. 1988), fatty acids (Sjögren et al. 2003), proteinaceous compounds (Magnusson and Schnürer 2001), and cyclic dipeptides (Ström et al. 2002) were shown to control mold growth either on their own or synergistically. Most antifungal metabolites produced by LAB were described for strains isolated from cereals and sourdough (Corsetti et al. 1998; Lavermicocca et al. 2000; Rouse et al. 2008; Gerez et al. 2009). Various LAB strains selected by their *in vitro* antifungal properties were used as starter cultures in dough fermentations. *Lact. plantarum* 21B produces two antifungal compounds, phenyllactic and 4-hydroxy-phenyllactic acids, which retained their fungicidal activities after baking and prolonged the shelf life of bread (Lavermicocca et al. 2003). Dal Bello et al. (2007) reported that the outgrowth inhibition of *Fusarium* sp. in wheat bread by *Lact. plantarum* FST 1.7 was due to lactic acid, phenyllactic acid, and two cyclic dipeptides (cyclo L-Leu-L-Pro, and cyclo L-Phe-trans-4-OH-L-Pro). It remains to be determined whether sourdough cul-

tures or their filtrates are more effective as antifungal agents.

The officially authorized maximal concentration for calcium propionate (CP), which is 0.3%, is not effective in preventing several spoilage fungi (Lavermicocca et al. 2000). Thus, new preservation systems including antifungal LAB strains, in combination with stabilizer agents, may be a promising bio-strategy for controlling mold growth (Ryan et al. 2008). A proper combination of several antifungal LAB strains reduced by 50% the concentration of CP in wheat dough that is necessary to attain a bread shelf life similar to bread elaborated with 0.4% CP (Gerez et al. 2009).

Reduction of bread spoilage represents a challenging issue for the bakery industry; economic losses from bread due to fungal spoilage, and consumer demand for free-additive foods increase the need to seek for natural alternative preservation systems. The addition of antifungal lactic starter will allow reducing the amount of chemicals needed to assure the safety and quality of bread.

## 15.6. Phytase Activity

Wholemeal bread is a staple food in many countries because whole cereal flours provide fiber, complex carbohydrates, proteins, vitamins, and minerals. However, the presence of significant amounts of phytate in flours and wholemeal breads interferes with mineral absorption. Phytic acid (myo-inositol hexaphosphate) is considered an anti-nutrient that forms precipitate complexes with essential dietary minerals (calcium, magnesium, iron, and zinc), strongly decreasing their bioavailability (Maga 1982; Torre et al. 1991). These complexes may also interfere with enzymatic degradation of proteins (Greiner and Konietzny 2006). Phytic acid is dephosphorylated by phosphatase enzymes or phytases (EC 3.1.3.26, myo-inositol hexakisphosphate phosphohydrolase), which hydrolyze phytic acid into inositol and inorganic phosphate and are widely distributed in plants, animal tissues, and microorganisms. Many LAB strains have shown phosphatase activity, and the microbial source of phytase is the most promising source for cereal-based foods

(De Angelis et al. 2003). The role of LAB in the degradation of phytate during lactic acid fermentation in general, and during sourdough fermentation in particular, was reported by Reale et al. (2007). The authors pointed out that LAB provide favorable conditions for the endogenous cereal phytase activity by lowering the pH value. The observed reduction in phytate content during lactic acid fermentation might therefore be due to an activation of endogenous plant phytases or coprecipitation of phytate and proteins as a consequence of pH fall during fermentation.

### 15.7. New Perspectives

Different projects are ongoing to evaluate the possible role of cereals as substrates for the development of novel functional foods due to the non-digestible components of the cereal matrix and by adding selected LAB strains (Charalampopoulos et al. 2002, 2003). In this new trend, the composition and processing of cereal grains; substrate formulation; growth capability and productivity of starter cultures; stability of probiotic strains during storage; and organoleptic properties and nutritional value of the final product are key parameters to be considered (Charalampopoulos et al. 2002). Cereal constituents such as starch can be used as encapsulation materials for probiotics to improve their stability during both storage and passage through the gastrointestinal tract (Capela et al. 2005; Ding and Shah 2007). Moreover, probiotics must meet the criteria not only for good survival during the process but also for fermentation and symbiosis with other starter cultures used (Kedia et al. 2007). Nowadays, the use of probiotic bacteria as starter cultures and as bio-preservatives in baked goods is at its early stage and merits further investigation (Vogel et al. 2002; Gerez et al. 2009). Some new cereal-based fermented foods are considered probiotic products; for example, Ogi with improved characteristics, named Dogik, has been developed using lactobacilli isolated from African fermented foods with strong antibacterial activity against some diarrheagenic bacteria (Okagbue 1995). Another example is Yosa, which is a new snack food made from oat bran pudding

cooked in water and fermented with LAB and bifidobacteria. After fermentation, the matter is then flavored with sucrose or fructose and fruit jam. It is mainly consumed in Finland and other Scandinavian countries (Wood 1997; Salminen et al. 1998).

### 15.8. Conclusions

The multiple beneficial effects of cereals together with LAB can be exploited in different ways, leading to the design of novel cereal-based foods or cereal ingredients that can target specific populations. However, the development of new processing technologies to enhance the health potential and acceptability of cereal-based products is of major importance constituting a trend that is very likely to continue.

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## Chapter 16

# An Overview of Lactic Acid Bacteria Applications for Healthful Soy Foods Development

Graciela Savoy de Giori, Laura Aguirre, José Marazza, and Marisa S. Garro

*The food industry is in continuous transition and is nowadays adopting an innovative and market-oriented position. This transformation is accompanied by an increasing demand for high-value-added products that meet consumers' demand for superior taste and convenience, health, and safety. The increased interest in adopting healthy eating habits to prevent certain diseases has led to the study and development of new functional foods. Food-grade fermentation can provide ingredients with new functionalities, thus constituting a cost-effective manufacturing process that uses microbial bioconversions to produce functional metabolites. Soy-derived foods, a versatile and a rich source of essential nutrients, have recently received much attention for their preventive effects on certain chronic diseases. Lactic acid fermentation, one of the most practical methods of food preservation, is able to enhance the nutritional and sensorial characteristics of fermented soy products. In recent years, selected lactic acid bacteria strains have been thoroughly investigated for specific health effects. In this chapter, health-promoting properties of fermented soy food obtained either directly by live LAB or indirectly as result of biogenic microbial metabolites derived from fermentation processes will be discussed with special emphasis on new starter cultures with novel properties to help the production of tastier, safer, and healthier soy products.*

### 16.1. Introduction

Recently, there has been an increased interest in adopting healthy diets to prevent diseases, and, as a

consequence, the so-called functional foods market has gained great importance. At present, the use of functional starter cultures in the functional food fermentation industry is being explored. Lactic acid bacteria (LAB) are able to produce antimicrobial substances, useful enzymes, or nutraceuticals, among other metabolites. LAB displaying health-promoting properties provide novel and attractive food products to the consumers. These types of foods are produced from a variety of food substrates worldwide. The implementation of carefully selected starter cultures in fermentation processes can help to achieve the *in situ* expression of the desired property, maintaining a perfectly natural and healthy product.

Soybean, one of the most important grains of the legume family and a major protein source for livestock, is relevant from both economical and nutritional standpoints. This review describes the challenges associated with the scientific development of fermented functional soy foods, emphasizing the beneficial effects of LAB.

### 16.2. Soybean Nutritional Components

Although the nutrient content of soybeans differs widely due to variety diversity and growth conditions, soybeans typically contain 35%–45% protein, 20%–30% lipids, 26%–30% carbohydrates, 10%–13% moisture, and around 5% minerals and ash. Because soy protein contains most of the essential amino acids for human nutrition, it is an important substitute for animal protein and constitutes a good alternative for lactose-intolerant or cow milk



allergic people. These reasons have significantly increased the use of soy in human nutrition.

Numerous products that are currently available in the market are based on soy or contain soy ingredients. Soy foods, also known as oriental soy products, are typically divided into two classes: non-fermented and fermented products. Among non-fermented soy foods, tofu is the most popular, followed by soymilk and soy sprouts, whereas soy sauce, miso, tempeh, and natto are the most typical among fermented soy products. Fermented soy foods vary greatly in preparation methods, microorganisms involved and utilization, all of them being mainly fermented by fungi except for natto, which results from bacterial fermentation. However, LAB also play an important role in the late fermentation stages of these products. LAB are also used in the production of fermented dairy analogs, that is, acidophilus soymilk, cultured butter soymilk, and soy cheese, using soybean as a substrate. Their microbial action contributes beneficially to both processing technology and quality of the end-product in terms of flavor, shelf life, and nutritional value.

### 16.3. Soy Carbohydrates Metabolism by LAB

Mature soybeans possess single sugars (monosaccharides and disaccharides), polysaccharides, and oligosaccharides. Among these, the main sugars are raffinose and stachyose, which contain one sucrose moiety linked by  $\alpha$ -1,6 bonds to one (raffinose) or two (stachyose) units of galactose. These soluble carbohydrates have received more attention because their presence has been linked to flatulence and abdominal discomfort associated with soybeans and soy products. In fact, flatulence is one of the major factors limiting the use of soybean as food (Steggerda et al. 1996). This problem has been noted most frequently with soy products from which carbohydrates have not been removed or degraded.

Humans are not endowed with the  $\alpha$ -galactosidase ( $\alpha$ -Gal) enzyme in the gut that is necessary for hydrolyzing the  $\alpha$ -galactosidic linkage present in these oligosaccharides ( $\alpha$ -GOS). When consumed by humans,  $\alpha$ -GOS cannot be digested in the duo-

denal and small intestinal mucosa. Thus, intact sugars pass directly to the lower intestine, where they are degraded by microorganisms such as *Clostridium* and *Bacteroides*, resulting in the production of gases such as hydrogen, methane, carbon dioxide, and nitrogen. Consequently, the host experiences flatulence and undesirable side effects (Rackis 1981; Liener 1994). Therefore, removal of  $\alpha$ -GOS from soy foods has been a major objective to improve their nutritive value. Many attempts have been made to eliminate  $\alpha$ -GOS from soybeans.  $\alpha$ -GOS are heat stable, so several alternative approaches to heat treatments have been sought. Although physical methods such as bean soaking, bean germination, water extraction, and ultrafiltration are successful, they are tedious (Ibrahim et al. 2002). Alternatively, the use of the enzyme  $\alpha$ -Gal to remove  $\alpha$ -GOS from leguminous seeds has been proposed as a biotechnological approach (Ganiats et al. 1994; Slominski 1994).

The enzyme  $\alpha$ -Gal ( $\alpha$ -D-galactoside-galactohydrolase, EC 3.2.1.22) catalyzes the hydrolysis of  $\alpha$ -1–6-galactoside links that are present in  $\alpha$ -GOS such as raffinose. Fermentation with fungi, yeasts, and bacteria displaying  $\alpha$ -Gal activity to reduce  $\alpha$ -GOS levels in soybeans has been attempted over the years (Cruz et al. 1981; Rehms and Barz 1995; Gote et al. 2004).  $\alpha$ -Gal has been isolated from plant and microbial sources with markedly different properties according to the origin (Kotwal et al. 1998; Gote et al. 2004). This enzyme is generally produced in culture conditions supplemented with one or more  $\alpha$ -D-galactopyranosyl groups as carbon source (Gote et al. 2004). Several yeast strains are known to produce high levels of extracellular  $\alpha$ -Gal activity, suggesting its potential industrial application for the improvement of the nutritional value of soybean by reducing the  $\alpha$ -GOS content (Viana et al. 2006). However, no reliable, inexpensive, and efficient enzymatic process with native or recombinant yeast enzymes is available so far. The potential enzymes suggested for this purpose are generally of microbial origin and do not have the Generally Recognized as Safe status.

Indeed, the use of LAB with  $\alpha$ -Gal activity for fermented soy products is promissory and attractive.

Several LAB are known to produce  $\alpha$ -Gal (Garro et al. 1996; Gopal et al. 2001; Silvestroni et al. 2002). This enzyme is inducible by  $\alpha$ -GOS and is mostly localized in the cytoplasm in most microorganisms (Garro et al. 1996; Gote et al. 2004). Recently, Yoon and Hwang (2008), while studying  $\alpha$ -Gal distribution in *Lactobacillus curvatus* and *Leuconostoc mesenteroides* strains found  $\alpha$ -Gal activity in both intracellular and extracellular fractions, with the former showing higher  $\alpha$ -Gal activity. *Lactobacillus* species are also able to use  $\alpha$ -GOS as an energy source to grow in soymilk. *Lactobacillus helveticus* (Angeles and Marth 1971; Murti et al. 1993), *Lactobacillus fermentum* (Garro et al. 2004), *Lactobacillus reuteri* (Tzortzis et al. 2004), *Lactobacillus acidophilus* (Shelef et al. 1998; Wang et al. 2002, 2003), and *Lact. curvatus* (Yoon and Hwang 2008) are the main lactobacilli that use  $\alpha$ -GOS and grow well in soy beverages.

Several studies have focused on the molecular characteristics of the  $\alpha$ -Gal genes expressed by microorganisms, along with the kinetic properties of the gene products (Schuler et al. 1985; Sripuan et al. 2003; Kim et al. 2005). The first characterization of the  $\alpha$ -Gal gene *mela* from lactobacilli, specifically from *Lactobacillus plantarum* ATCC 8014, was reported by Silvestroni et al. (2002). The *mela* gene is surrounded by genes involved in  $\alpha$ - and  $\beta$ -galactoside utilization and is transcribed from its own promoter. Transcription of *mela* is independent of surrounding genes and is induced by melibiose and partially repressed by glucose. A putative galactoside transporter termed RafP with high homology to *LacS*, the only transporter for both  $\alpha$ - and  $\beta$ -galactosides in *Streptococcus thermophilus*, was found upstream of *mela*. The *lacL* and *lacM* genes that encode a heterodimeric  $\beta$ -Gal were identified downstream of *mela*. A putative *galM* gene was identified in the same cluster, suggesting the presence of a galactose operon. These results indicate that the genes involved in galactoside catabolism are clustered in *Lact. plantarum* ATCC 8014. Subsequently, the characterization of genes involved in  $\alpha$ -GOS hydrolysis by *Lactococcus raffinolactis* was described by Boucher et al. (2003). In this species, the  $\alpha$ -Gal metabolism was driven by an

operon that contains the three genes *aga*, *galK*, and *galT*. The *aga* gene encodes  $\alpha$ -Gal, while *galK* and *galT* most likely encode two enzymes of the Leloir pathway (the enzymes galactokinase and galactose 1-phosphate-uridylyltransferase, respectively).

Carrera-Silva et al. (2006) reported the genetic organization of the *mela* locus in *Lact. fermentum* CRL722, which resembles the one previously studied in *Lact. plantarum* (Silvestroni et al. 2002), except for *LacI*, which is replaced by  $\beta$ -Gal-encoding genes in *Lact. plantarum*.

The phylogenetic tree obtained from the amino acid sequences of  $\alpha$ -Gal from different species (NCBI, BLASTp databases) showed that  $\alpha$ -Gal from *Lact. fermentum* CRL722 does not seem to be related to those from other bacteria. The relationship of this enzyme with those present in other lactobacilli seem to be similar to those of the genera *Geobacillus*, *Thermoanaerobacter*, and *Clostridium*. The genus *Bifidobacterium* shows the same level of kinship as some fungi of the genus *Aspergillus*, but both seem to be far relatives of *Lact. fermentum* CRL722.

Engineered LAB have been constructed to degrade  $\alpha$ -GOS. For this purpose, the  $\alpha$ -Gal structural gene from *Lact. plantarum* ATCC 8014 was cloned and expressed cytoplasmically in *Lactococcus lactis* cells used as bacterial vehicle. The recombinant strains were tested *in vivo* using an animal model to reduce  $\alpha$ -GOS levels. The cytoplasmic form proved to be very promising because  $\alpha$ -Gal-overproducing *L. lactis* was lysed in the duodenum and released large quantities of this enzyme in the upper intestinal tract with the concomitant reduction of  $\alpha$ -GOS content (Connes et al. 2004; LeBlanc et al. 2004). However, the use of such bacteria may depend on still controversial decisions about the acceptability of genetically modified organisms in nutrition (see Chapter 20).

A specific strain is considered efficient in the prevention of digestive disorders associated with  $\alpha$ -GOS consumption if it is able to degrade these undesirable sugars in the upper digestive tract (duodenum, jejunum, or ileum) and to prevent their arrival at the large intestine where they are fermented by endogenous microbiota. LeBlanc et al.

(2005) found evidence of short-lived  $\alpha$ -Gal activity in the stomach and in the small intestinal chyme when conventional rats were given live or cell-free extracts of *Lact. fermentum* CRL722, suggesting that this activity could significantly reduce the physiological effects associated with  $\alpha$ -GOS consumption. These observations were confirmed by the same authors (LeBlanc et al. 2008), who described an animal model that enables the elucidation of both the mechanism and parameters (i.e., influence of the LAB vehicle and administration mode) that influence the *in vivo* expression of LAB enzymatic activities. Non-recombinant *Lact. fermentum* CRL722 co-administered with unfermented soymilk produced significant reduction (50%) in  $H_2$  emission, showing that  $\alpha$ -Gal from *Lact. fermentum* CRL722 remained active *in situ* in the gastrointestinal tract of rats monoassociated with *Clostridium butyricum*. In human microbiota-associated rats, *Lact. fermentum* CRL722 also induced a significant reduction in  $H_2$  emission (70%). This was the first study to demonstrate the efficiency of  $\alpha$ -Gal-producing lactobacilli in improving the digestibility of  $\alpha$ -GOS by reducing gas production *in situ*.

#### 16.4. Soy Protein Metabolism by LAB

Soybean proteins generally constitute about 35% to 45% of total seed on a dry basis. Globulins represent around 90% of total soybean, among them glycinin and  $\beta$ -conglycinin are two major storage globulins. Glycinin, also called soy 11S protein, consists of acidic and basic polypeptides linked by a disulfide bridge.  $\beta$ -Conglycinin, a 7S protein, is a trimeric glycoprotein consisting of three types of subunits:  $\alpha'$ ,  $\alpha$ , and  $\beta$  (Hou and Chang 2004a, 2005b).

Soybeans provide high-quality proteins, which are also a potential source of bioactive peptides. Enzymatic hydrolysis is a commonly used method for releasing bioactive peptides from their protein precursors and for producing food-grade protein hydrolysates. Evidence has shown that fermentation processes affect the nutritional quality of legumes by improving protein content and digestibility as a

consequence of the partial degradation of complex stored proteins into more simple and soluble products (Frias et al. 2008).

Despite the mentioned advantages of soy products, they can cause allergies in certain people. Soybean allergy is considered one of the most common allergies among children. Intolerance to soy protein may cause different clinical syndromes such as rhinitis, urticaria, asthma, atopic dermatitis, anaphylactic shock, or even death (Table 16.1). Allergy to soy resides in its protein fractions, 7S and 11S globulins being the most important. At least 21 immunoglobulin E (IgE)-binding soy proteins, with molecular masses ranging from 7.5 to 97 kDa, may be involved in clinical allergy (Wilson et al. 2005; Ogawa 2006).

Food processing including both thermal and enzymatic treatment may decrease the allergenicity of proteins because alteration of protein conformation by heating has been associated with loss of conformational epitopes and consequent reduction or abolition of allergenic potential of proteins (Thomas et al. 2007). In fact, the degradation of soybean allergens during fermentation by microbial proteolytic enzymes in soy sauce, miso, soybean ingredients, and feed-grade soybean meals has been demonstrated (Kobayashi 2005; Frias et al. 2008). The immunoreactivity of soy products is affected by the type of microorganism used during the fermentation process. Specifically, mold proteolysis in natural fermented cracked seeds and flour products was less efficient than bacterial proteolysis in eliminating immunoreactive proteins, probably due to the slower growth rate of viable mold during the fermentation process (Frias et al. 2008). When the same fermentation conditions were applied to solid fermented products, faster microbial growth rate and better efficacy in the breakdown and transformation of high-molecular-weight proteins and peptides were found. Thus, *Lact. plantarum* exhibited a better potential for developing reduced immunoreactive products as compared with mold strains. Recently, Aguirre et al. (2008) reported that fermentation processes using LAB are able to decrease or eliminate antigenic soybean proteins and, at the same time, improve the nutritional value of the final

**Table 16.1.** Soybean proteins allergen and their clinical relevance.

Protein fraction	MW (kD)	Clinical effect	References
Trypsin inhibitor (Kunitz)	20	Asthma	Ogawa et al. (1991)
Ber e 1; Albumine-2S (transgenic)	17	Anaphylaxis	Ogawa et al. (1991)
Cotyledonous tissue Gly m4 (SAM22-H4 gene family)	—	Cross-reactions with birch pollen	Mittag et al. (2004)
Soy whey fraction	18–21, 29–31	—	Ogawa et al. (1991)
Storage protein			
Vacuoles P39-1 polypeptide	39	Skin allergy	Xiang et al. (2008)
Glycinin G1 (Acidic subunit 11S)	35–40	Children's allergy	Beardslee et al. (2000); Xiang et al. (2002)
Glycinin G2 (Basic subunit 11S)	22	Allergy	Helm et al. (2000a,b); Xiang et al. (2002)
$\beta$ -conglycinin (7S-Globulin)	33–55 <sup>a</sup>	Atopic dermatitis	Ogawa et al. (1991)
Gly m Bd 28K	28	Atopic dermatitis	Tsuji et al. (1997, 2001); Xiang et al. (2004); Hiemori et al. (2000, 2004)
Gly m Bd 30K (P34 Thiol-protease)	34	Atopic dermatitis	Ogawa et al. (1991)
Subunit $\beta$	42	Allergy	Ogawa et al. (1991)
Gly m Bd 60K (Subunit $\alpha$ )	63–67	Allergy	Rihs et al. (1999); Ogawa et al. (1991)
Gly m Bd 60K (Subunit $\alpha'$ )	71	Allergy	Rihs et al. (1999); Ogawa et al. (1991)
Hull protein (Gly m 1.0102)	7.0	Asthma	Rodrigo et al. (1990); González et al. (1992)
Hull protein (Gly m 1.0101)	7.5	Asthma	Rodrigo et al. (1990); González et al. (1992)
Hull protein (Gly m 2)	8.0	Asthma	González et al. (1992)
Gly m 3 (Profilin)	14	Allergy	Rihs et al. (1999)
Gly m Bd 50K	50	Asthma, rhinitis	Codina et al. (2002)

<sup>a</sup>Fractions: 33–35/35–38/40–41/47–50/52–55.

fermented soybean food. This approach may be a valuable tool for developing hypoallergenic soy food products.

Peptides with biological activities released from soy protein breakdown have attracted the attention of the research community in recent years (Gibbs et al. 2004; Wang and Gonzalez de Mejia 2005). It is well known that during gastrointestinal digestion or food processing, these peptides are released from the parent protein acting as regulatory compounds with hormone-like activities. Thus, soy protein consumption reduces clinical and biochemical abnormalities in diseases mediated by lipid disorders (Torres et al. 2006). Studies on cell cultures demonstrated that soybean 7S globulin and peptides derived from this protein are able to upregulate low-density lipoprotein (LDL)-receptor activity (Lovati et al. 2000; Manzoni et al. 2003).

Acidic and enzymatic hydrolyses are two important methods for generating soybean peptides. Acid hydrolysis is relatively simple and less expensive, but it is difficult to control and amino acid damage

may occur. Enzymatic hydrolysis is easy to control and uses milder conditions. Thus, fermentation is an efficient way to produce bioactive peptides, which can be released by microbial activity during food fermentation or through enzymes derived from microorganisms (Korhonen and Pihlanto 2003). Interestingly, depending on the initial protein source and the enzymes used, peptides released may display different biological activities, that is, antioxidant, antimutagenic, antihypertensive, hypocholesterolemic, immunomodulatory, and so on (Table 16.2). Several angiotensin I-converting enzyme (ACE) inhibitory peptides have been found in enzyme hydrolysates of soy proteins. Chen et al. (2003, 2004) identified ACE inhibitory peptides in the peptic digest of soybean protein. Although soy protein breakdown by “commercial enzymes” or enzymes from microorganisms such as *Mucor* sp., *Aspergillus oryzae*, *Rhizopus* sp., *Bacillus natto*, or *Bacillus subtilis* is well documented (Gibbs et al. 2004; Wang and Gonzalez de Mejia 2005), the action of proteolytic enzymes from LAB on soy

**Table 16.2.** Bioactive peptides derived from soy hydrolysate and soy-fermented products.

Substrate	Enzymatic source	Activity	References
Soybean	<i>B. subtilis</i> protease	Antihypertensive	Kitts and Weiler (2003)
	Pepsin	ACE inhibitory	Chen et al. (2003, 2004)
	Thermolysin	Hypocholesterolemic	Yoshikawa et al. (2000)
	Fungi fermentation	Antimutagenic	Hung et al. (2007)
Soy protein	Commercial enzymes <sup>a</sup>	Antihypertensive, Antioxidative	Korhonen and Philanto (2003)
	Alcalase	Hypocholesterolemic	Zhong et al. (2007)
	Soybean protease	Antihypertensive	Kodera and Nio (2006)
Glycinin soybean	Protease	ACE inhibitory	Gouda et al. (2006)
$\beta$ -conglycinin	Thermolysin	Immunomodulatory	Yoshikawa et al. (2000)
Soy protein (native or heated)	Commercial enzymes <sup>b</sup>	Antioxidative	Peña-Ramos and Xiong (2002)
Commercial soy paste	Fermentation	Antihypertensive	Shin et al. (2001)
Soy milk	LAB <sup>c</sup> and <i>Aspergillus</i>	ACE inhibitory	Tsai et al. (2006)
	LAB <sup>d</sup>	Antioxidant	Wang et al. (2006)
Soy yogurt	LAB <sup>e</sup>	ACE inhibitory	Donkor et al. (2005)
Tofuyo	Fermentation	ACE inhibitory	Kuba et al. (2003)
Defatted soy meal	Alcalase	Antihypertensive	Wu and Ding (2001)
	Thermolase	Anticancer	Kim et al. (2000)
Digested natto/digested tempeh	Commercial protease	Antioxidant, Antithrombotic, ACE inhibitory, Surface tension	Gibbs et al. (2004)

<sup>a</sup> Alcalase® (Novo Nordisk Biochem Inc., China), Proteinase S (Amano Seiyaku Co., Nagoya, Japan), and Trypsin (Amano Seiyaku Co., Nagoya, Japan).

<sup>b</sup> Papain, pepsin, and chymotrypsin and Alcalase® (endoproteinase from *Bacillus licheniformis*), Protamex™ (*Bacillus* protease complex), and Flavourzyme™ (endoprotease and exopeptidase from *Aspergillus oryzae*) (all from Novo Nordisk, Inc., Bagsvard, Denmark).

<sup>c</sup> *Lact. acidophilus*, *Lact. casei*, *S. thermophilus*, and *Lact. bulgaricus*.

<sup>d</sup> *Lact. acidophilus*, *Bifidobacterium* ssp., and *S. thermophilus*.

<sup>e</sup> *Lact. acidophilus*, *Bifidobacterium lactis*, *Lact. paracasei*, *S. thermophilus*, and *Lact. Bulgaricus*.

protein extract (SPE) has not been thoroughly studied and knowledge is still limited.

The action of whole LAB cells on SPE as an attempt to gain better knowledge of their hydrolytic ability and the nature of the generated products was evaluated (Aguirre et al. 2008). The proteolytic activity of different LAB species (*Lactobacillus paracasei* subsp. *paracasei*, *Lact. fermentum*, *Lactobacillus delbrueckii* subsp. *lactis*, *Lact. plantarum*, *Lact. helveticus*, *Lact. reuteri*, and *Pediococcus pentosaceus*), assayed on SPE at 37°C for 6h, was evaluated by SDS-PAGE, reverse-phase high-performance liquid chromatography (HPLC), and free amino acid analyses. Results showed that  $\alpha$ - $\alpha'$  subunits of  $\beta$ -conglycinin were the preferred substrates for most LAB. Only a few strains exerted action against the basic polypeptides of glycinin, which is the least degraded of all soy protein frac-

tions. Different profiles of hydrophilic and hydrophobic peptides from soy protein fractions were generated by the studied LAB strains. These strains also increased the level of total free amino acids in the SPE and hydrolyzed principally essential and flavor precursor amino acids.

The role of free radicals and active oxygen species in various diseases including aging, cancer, inflammation, and the toxicity of numerous compounds is well documented (Middleton et al. 2000; Cadenas and Parker 2001). To replenish the aging-induced loss of defense systems against oxidative stress, the human body must be provided with a constant supply of antioxidants through a proper diet. Several food proteins possess antioxidant properties, and hydrolysis of these proteins may release peptides and amino acids with proven antioxidant activity. Amino acids such as Tyr, Met, His, Lys,



and Trp have been shown to display antioxidant capacity (Chen et al. 1998; Saito et al. 2003; González de Mejía and de Lumen 2006). This activity is measured indirectly by the effects of antioxidants in controlling the extent of oxidation. Some methods show the advantage of determining the antioxidant capacity as a global characteristic of the product that can be used to characterize the raw material and its evolution during processes (Wang and Gonzalez de Mejia 2005). During hydrolysis, the soy protein structure is altered, and more active amino acid R groups are exposed, suggesting that soybean peptides can have higher antioxidant activity than intact protein. Thus, it was shown that enzyme digestion of  $\beta$ -conglycinin and glycinin possessed increased (3–5-fold) radical scavenging activities (Chen et al. 1998).

The antioxidant capacity of peptides released from soy proteins by the LAB proteolytic system has also been reported. Wang et al. (2006) showed that the antioxidant activity of soymilk fermented with mixed LAB cultures plus bifidobacteria for 24 h was higher than that fermented with bacterial culture individually. Moreover, antioxidant activity increased when the fermentation period is prolonged (Aguirre et al. 2008). Soy protein hydrolysates obtained from *Lact. paracasei* subsp. *paracasei* CRL207 on SPE incubated for 6 h and then fractionated by HPLC showed 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity in fractions with retention time (RT) of 19.8, 25.89, and 46.30 min (Aguirre et al. 2008). The DPPH radical is a stable free radical compound with strong absorbance at 517 nm. The peptide fraction with an RT of 46.30 min was more effective in suppressing oxidation in food and biological systems only when oxidation was induced by DPPH radical-like substances.

### 16.5. Isoflavones Transformation by LAB

Soy isoflavones belong to a subclass of flavonoids often called phytoestrogens because of their ability to interact with estrogen receptors in cells. Their basic structural unit comprises two benzene rings linked via a heterocyclic pyrone ring. These phytoestrogenic isoflavones are of great interest because

of their potential protective or preventive activity against a number of diseases such as cardiovascular diseases, cancer, and osteoporosis (Tsangalis et al. 2005).

Worldwide, soybeans are the almost exclusive dietary source of isoflavones, which are also present in unfermented soy foods, occurring in glucose-conjugated (genistein, daidzein, glycitin) (Coward et al. 1993; Wang and Murphy 1994) or acetyl- and malonyl- $\beta$ -glucoside forms, which are polar and water-soluble compounds (Coward et al. 1998; Song et al. 1998). Once ingested, isoflavone glucosides are hydrolyzed to aglycones, which are absorbed more quickly than their respective glucosides.

The concentration and composition of soy isoflavones in soy foods and soy-containing foods vary markedly. Isoflavone content of some representative soy foods is shown as follows: soybean (0.60–2.39 mg/g), soy flour (0.60–2.35 mg/g), soy protein (0.45–2.00 mg/g), soymilk (0.01–0.31 mg/g), tempeh (0.43–0.63 mg/g), tofu (0.10–0.50 mg/g), and miso (0.20–1.00 mg/g) (U.S. Department of Agriculture 2002). These differences may result from formulation variability, changes in raw materials used and processing methods and techniques, as well as post-processing changes during distribution and storage. Wang and Murphy (1994) and Song et al. (1998) reported that total isoflavone concentration of non-processed soybeans was in the range of 1.2–4.2 mg/g.

Certain processing techniques such as hydrolysis, fermentation, and foam removal can influence the isoflavone content and isomer composition of soy products. Thus, soy flour and texturized soybean proteins contain between 1.1 and 1.4 mg/g, whereas both, soy concentrates, produced by alcohol washing and second generation soy foods, such as tofu, soy-yogurt, and tempeh burger, have lower isoflavone concentrations (Wang and Murphy 1994).

Kao et al. (2004) reported high aglycone concentrations in soymilk or tofu while a reversed trend was found for the glucoside isoflavones when the soaking temperature of the process was increased. However, Shimoni (2004) stated that isoflavones are generally stable through heating, although they may intraconvert between different forms. Thus, baking soy-enriched bread at 165°C for 50 min resulted in stability of the total isoflavone content (Coward

et al. 1998), while daidzein and genistein conjugates released free isoflavones after heating under acid conditions (Wang et al. 1990). Other studies have shown that the overall content of isoflavones in soy protein isolates and corn mixtures decreased at high temperatures (from 110 to 150°C; Mahungu et al. 1999). Most of the studies just mentioned were limited to total isoflavone losses during processing at high temperatures. Ungar et al. (2003) characterized the kinetics of daidzein and genistein degradation in model solutions (pH 7 and 9), confirming that they were first-order reactions. Genistein degradation was accelerated in an alkaline environment, while a higher stability at pH 7 was also detected in daidzein solutions. Overall, degradation rates of daidzein were higher than that of genistein, demonstrating again that daidzein is certainly more labile to degradation than genistein at high temperatures.

In soybean and non-fermented soy foods (e.g., tofu), isoflavones are found as glucoside conjugates (genistin, daidzin, glycitin), which comprise 80% to 95% of the isoflavone concentration (Coward et al. 1993; Wang and Murphy 1994; Lori et al. 1998; Song et al. 1998), while fermented products (e.g., tempeh) are rich in aglycones (Wang and Murphy 1994; Coward et al. 1998). It has been shown that the glucoside isoflavones are very poorly absorbed in the small intestine compared with their aglycones because the former have greater molecular weight and higher hydrophilicity (Chang and Nair 1995). Since the structure itself is a limiting factor for absorption in the gastrointestinal tract (Hendrich et al. 1999), chemical forms of isoflavones and their metabolites influence the extent of absorption; aglycones are more readily absorbed and more bioavailable than highly polar conjugated species (Setchell 2000). Therefore, the glucoside forms must be first hydrolyzed by intestinal  $\beta$ -glucosidases ( $\beta$ -glu), which deconjugates the pran ring of isoflavone and the sugar moieties to yield aglycone forms that can be absorbed *in vivo*. This fact (and some *in vivo* results showing superior estrogenic effects of genistein over its glucosides) has led to the development of aglycon-enriched products either by  $\beta$ -glu treatments (Park et al. 2002; Otieno and Shah 2007) or by fermentation (Otieno and Shah 2007).

In this sense, LAB could increase aglycone concentration in soy food manufacture because certain strains possess  $\beta$ -glu activity. De Boever et al. (2001) reported that semi-skimmed milk with soy germ powder fermented by *Lact. reuteri* generated isoflavone aglycones through hydrolysis of the  $\beta$ -glycosidic bonds. Wei et al. (2007) also showed that one strain of *Lact. paracasei*, two strains of *Lact. acidophilus*, and one strain of *Bifidobacterium longum*, used singly or in mixed cultures, were able to increase the isoflavone aglycone contents in fermented soymilk; addition of sugar to the substrate did not enhance isoflavone aglycone concentrations. Otieno et al. (2006) found that the hydrolytic potential of  $\beta$ -glu activity of *Bifidobacterium*, *Lact. acidophilus*, and *Lact. casei* strains for biotransformation of isoflavone glucosides in soymilk was strain-dependent. Recently, Marazza et al. (2009) reported that *Lact. rhamnosus* and *Lact. paracasei* subsp. *paracasei*, among seven species of lactobacilli analyzed, were able to express  $\beta$ -glu activity upon its specific substrate (pNPGlu), *Lact. rhamnosus* strains showing higher specific  $\beta$ -glu activity than *Lact. paracasei* subsp. *paracasei*. The isoflavone biotransformation by  $\beta$ -glu activity showed a correlation between their specificity and the temperature and incubation time. In addition, Chun et al. (2008) also showed that *Weissella* strains with  $\beta$ -glu activity isolated from human feces could effectively convert isoflavone glucosides to aglycones in soymilk.

The biological activity of soy products may be improved through fermentation processes and the stability of bioactive components could be maintained during the storage period to confer health benefits to the consumer. Isoflavone degradation in soymilk depends on the storage time and temperature,  $\beta$ -glu enzyme stability at storage temperature being strain-dependent. Marazza et al. (2009) has shown that  $\beta$ -glu activity of different LAB exhibited better stability at low temperatures (4°C and -80°C). Thus, the selection of LAB microorganisms as starter cultures for soy-based functional foods will depend on the  $\beta$ -glu activity and its stability at the selected storage temperature. Therefore, studies to properly define storage conditions of the product to

guarantee minimal losses of the bioactive components should be considered.

## 16.6. Conclusions and Future Directions

This chapter reviews the versatility of LAB for healthful soy foods production. LAB, used as starter cultures or as probiotics, or their biogenic compounds, are responsible for the positive impact of fermented foods on consumers' health. However, further research needs to focus on the isolation of new strains with improved health-promoting properties or strains with new health-related activities to facilitate their exploitation for the design of novel functional food products to meet consumers' needs and requirements.

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## Chapter 17

# The Functional Role of Lactic Acid Bacteria in Cocoa Bean Fermentation

Luc De Vuyst, Timothy Lefeber, Zoe Papalexandratou, and Nicholas Camu

*This review covers the state of the art of the cocoa bean fermentation process, in particular how fermentation is determined by the basic raw material, the cocoa pulp-bean mass, and by environmental and processing conditions, and how fermentation in turn influences flavor and health-promoting constituents of the manufactured end-products, cocoa powder, and chocolate. Several reviews and books have been published on this topic during the last 50 years, in particular, but not exhaustive, Roelofsens; Carr; Lehrian and Patterson; Jones and Jones; Wood and Las; Lopez and Dimick; Schwan et al.; Gotsch; Fowler et al.; Pontillon; Knight; Schwan and Wheals; Bartley; Thompson et al.; Afoakwa et al.; and Beckett. To avoid overloading of the text with references, only in particular cases will be referred to these reviews and books and to references already included in these works, with the exception of the parts on microbiology.*

### 17.1. Introduction

#### 17.1.1. History

The secrets of the cocoa tree, *Theobroma cacao* L. (literally “food of the gods”), were first exploited around 4000 years ago in the tropical rainforest of Latin America. The fruits (cocoa pods) of this tree contain seeds (cocoa beans) that can be processed into cocoa powder, cocoa mass, and cocoa butter. Cocoa (beans) is responsible for the unique flavor and melt-in-the-mouth properties of chocolate. The

story of how chocolate grew from a local and ritual Mesoamerican beverage gift of the gods for the local people to a nutritious and medicinal product for wealthy Europeans during the initial cocoa trade to a delicious sweet enjoyed around the world encompasses many different cultures and continents, with Latin America, Asia, Africa (cocoa plantations), and Europe (cocoa trade and chocolate production) playing a major role (Coe and Coe 2007). Today, approximately two-thirds of the world’s cocoa, which is estimated at over 3.5 million tons, is produced in West Africa (70%), followed by Latin America (16%), and Southeast Asia (14%) (<http://www.worldcocoaoundation.org>). At present, the eight largest cocoa-producing countries (in order of annual production size) are Ivory Coast (40%), Ghana (20%), Indonesia, Nigeria, Cameroon, Brazil, Ecuador, and Malaysia, which represent 90% of the cocoa world production (<http://www.icco.org>). Virtually all major cocoa production over the past five decades has involved bulk (basic or ordinary) cocoa. There are, however, many other smaller producers, particularly of fine (flavor) cocoas, which constitute about 5% of the cocoa world trade. The best known producer is Nacional (Ecuador), which is the source of the fine-flavor cocoa called Arriba. Less known is Java Fine or Edel cocoa, a light-breaking cocoa from Java (Indonesia), which is mainly used for its color. About 90% of the world’s cocoa is grown by smallholders, often in less well-developed tropical regions of the world. Chocolate production is mainly concentrated in Europe and the United

States. Leading European producers are Germany, the United Kingdom, France, Italy, Belgium, the Netherlands, and Switzerland. Whereas Belgium is world famous for its extremely fine, excellent tasting and high-quality chocolate, French dark chocolate became renowned in the French gastronomy, and Swiss milk chocolate is appreciated worldwide for its seductive silky smoothness and purity.

### 17.1.2. Chocolate and Human Health

The growing interest in the relationship between foods and human health has given a new impetus to chocolate production since the 1990s. As consumers actively search worldwide for food that is not only nutritious and delicious but also carries some functional benefits for their health, superior chocolates have been developed with health-promoting properties. Indeed, scientific studies on cocoa and chocolate have revealed antioxidative, anti-atherogenic, anticarcinogenic, psychoactive, and mood-elevating effects of chocolate components resulting from their moderate consumption (Knight 1999; Wollgast and Anklam 2000a; Smit and Blackburn 2005; Ding et al. 2006). With a view to improving the nutritional profile of chocolate, which is a high-calorie food, a range of new products has been introduced into the market, such as organic chocolates, chocolates without added sugar, lactose-free chocolates, polyphenol-enriched chocolates, fiber-enriched and prebiotics-containing chocolates, and probiotic chocolates (Knight 1999; Nebesny et al. 2007; Beckett 2009).

## 17.2. Cocoa Crop Cultivation and Harvest

The cocoa tree is a small tree, cultivated worldwide as a shade crop in the rainforest in a 20° belt north and south of the equator (Wood and Lass 1985; Fowler et al. 1998; Bartley 2005). A large number of cocoa tree varieties exist, which are grouped according to morphological (fruits and seeds) and geographical (genetic origin) characteristics. This large cocoa variety is due to genetic factors (a vast

array of genotypes, interbreeding, etc.) and human intervention (agricultural practices, re-crossing, etc.). In principle, all cocoa varieties originate from the Criollo and Forastero varieties, which are considered indigenous to the Upper Amazon basin (Amazonian region, Venezuela). Of the Forastero varieties, the melon-shaped Amelonado (Lower Amazon) is the most widely grown (Brazil and West Africa). Trinitario, a third cocoa group often referred to in the literature, originates from Trinidad and has been so named to distinguish it from the native cocoa of Venezuela. It is actually a hybrid between Criollo and Forastero (most often Lower Amazon) varieties, which is particularly suitable for cultivation. Today, Criollo, Forastero, and Trinitario varieties represent approximately 1%, 94%, and 5% of the world production of cocoa. Criollo and Trinitario are considered fine cocoas that are often sold at higher prices than Forastero, and used for premium plain dark chocolates and couvertures. However, Forastero is much less prone to various diseases and overcomes several other weaknesses (yield, bearing, etc.), giving a more stable crop from year to year, and it is thus preferred by farmers throughout the world, yielding bulk cocoa (for milk and dark chocolates and other confectionary products). The exact origin of the Nacional type of cocoa is not known, but it is generally considered native to Ecuador. Molecular studies indicate that Nacional cocoa genotypes, first considered a Forastero variety, are genetically different from the other cocoa groups (Crouzillat et al. 1996). In general, many cocoa farmers grow several varieties of cocoa and, due to cross-pollination and re-crossing, single trees with all the characteristics of a specific cocoa type are rare. Moreover, despite its long history of cultivation and selection, current cocoa cultivars are quite similar to the originally cultivated cocoa germplasm. A vast body of work has been carried out to improve the cocoa crop, including germplasm collection, plant breeding, crop yield, rapid propagation, disease resistance, full-sun cultivation, pollination through insects, development of improved cocoa seeds, pods with more and bigger cocoa beans, beans with elevated fat levels, and so on. Unfortunately, probably not the best varieties of *T. cacao* have been planted world-

wide, as quality may have taken second place to quantity in the choice of tree. Each year, more than 20% of the cocoa production is lost to plant diseases caused by devastating fungal pathogens, viruses, and insects, and even greater losses will occur in the near future, due to further spread of important diseases among the monocultures, if the necessary precautions such as good agricultural practice and replanting are not carried out in time (<http://www.worldcocoaoundation.com>; <http://www.icco.org>).

Cocoa trees carry buds, flowers, and fruits at the same time. In general, thousands of flowers adorn the trunk and major branches of the cocoa tree. Only a few flowers will be fertilized through difficult cross-pollination, and no more than 1%–10% will develop into cocoa pods. The overall development of the pods takes 5–6 months from pollination to full ripeness. The oval, leathery cocoa fruits vary among varieties in size, shape, surface texture, and external color. They contain a pulpy endocarp (the cocoa pulp) in which the seeds (the cocoa beans) are embedded (Fig. 17.1). One flower yields about 20–40 seeds, depending on the cocoa variety, which are attached to a central core or placenta. Cocoa pods are indehiscent and do not abscise; hence, the fruits have to be cut down at harvest. At harvest time, cocoa pods change color from green or dark red-purple to yellow, orange, or red, depending on the cocoa variety. As the mucilage contains a germination inhibitor, germination of the seeds inside the pod is delayed; however, once the pod is opened, the mucilage decomposes rapidly and germination of the seeds begins under appropriate humidity and temperature conditions. Cocoa trees produce a few hundred to more than 3000 kg of dry beans per hectare, depending on cocoa variety and agronomic and farming practices. Healthy, ripe pods are harvested twice a year, representing a main-crop and mid-crop, with intervals of approximately 3 weeks within the peak seasons. It is common practice in many cocoa-producing regions to harvest the pods over 3–4 days, according to the cocoa yield and size of the farm, before the collected pods are transported to a place suitable for subsequent handling, either at the farm or at the edge of the plantation or at a processing plant.

### 17.3. The Cocoa Pulp or Fermentation Substrate

Ripe cocoa pods measure 10–35 cm long and weigh (wet) from 20 g to more than 1 kg. They contain a considerable amount of sweet, white, mucilaginous pulp surrounding the cocoa beans (Fig. 17.1). This cocoa pulp is a rich medium for microbial growth and hence forms the raw material for the actual cocoa fermentation. Sufficient pulp (mature pods) guarantees a correct fermentation process. The degradation of the mucilage during fermentation liberates pulp juices, referred to as “sweatings,” which drain away. Therefore, a correct harvest time of mature cocoa pods is of utmost importance. In over-ripe pods, the amount of pulp is too small and the pulp appears to be dry; underripe pods possess a dense pulp.

Cocoa pulp is composed of 82%–87% water, 10%–15% sugars, 1%–5% pectin, 1%–3% citric acid, 0.1%–0.4% other non-volatile acids (e.g., malic acid), 0.5%–0.7% proteins, and 8%–10% minerals and oligoelements (Lehrian and Patterson 1983; Schwan et al. 1995; Barel 1998). Of the sugars present, about 60% is sucrose and 39% is a mixture of glucose and fructose. The concentration of sucrose, glucose, and fructose is a function of cultivar and fruit age; unripe pods contain a higher proportion of sucrose, and ripe pods contain mainly fructose and glucose (Lehrian and Patterson 1983; Thompson et al. 2007). The amino acids glutamic acid and aspartic acid have been reported to be present at high levels in the cocoa pulp compared with other amino acids. Vitamin C is the most important vitamin in the cocoa pulp. Potassium and sulfate comprise the major cation and anion, respectively. The pH of the pulp is relatively low (pH 3.0–4.0), mainly due to its citric acid content. The high content of pectin and other polysaccharides (cellulose, hemicellulose, lignin) makes the pulp viscous, sticky, and cohesive.

### 17.4. Fresh, Unfermented Cocoa Beans

Fresh, unfermented cocoa beans are oval (2–3 cm long, 1.0–1.7 cm wide, 0.7–1.2 cm thick) and weigh



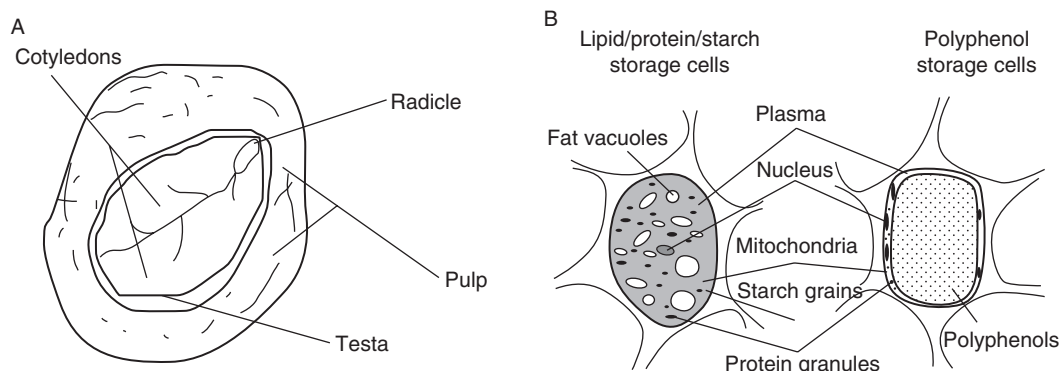
**Figure 17.1.** Opened cocoa pod with beans embedded in a mucilaginous pulp.

on average 2.5 g. The moisture content of the fresh beans is approximately 65%. The seeds basically consist of two parts: an outer part comprising the skin or testa (seed coat or shell), surrounding the bean, and an inner part comprising the embryo (germ) and two cotyledons contained within the testa and uniformed at a small embryonic axis (Fig. 17.2A). The cotyledons are referred to as the nibs in the cured beans. The testa of the cocoa seed is impermeable to large molecules (citric acid, poly-

phenols, alkaloids), whereas small volatile molecules, such as ethanol and acetic acid, can easily penetrate it. Hence, this biological barrier controls the kinetics of fermentation and concomitant diffusion processes (see below).

The cotyledons are basically made up of different types of storage cells, namely white lipid/protein/starch-containing cells and purple polyphenol-containing cells, with parenchyma plasma forming a grid between the two types of cells (Fig. 17.2B).





**Figure 17.2.** A. Cocoa bean. B. Cocoa bean storage cells.

Whereas the storage cells of starch granules, aleurone grains, and fat droplets constitute the reserve material for the embryo and lipids further form a natural barrier around the water-soluble compounds (enzymes, substrates, and inhibitors), the larger, vacuolated, polyphenol-storage cells contain unique cocoa bean components, including alkaloids (theobromine and caffeine) and polyphenols, both of which are possibly involved in stress resistance (light) responses of the plant.

The fat (cocoa butter) of the cocoa bean is important for chocolate production, as it will give chocolate its characteristic texture (Asep et al. 2008; Beckett 2009). Fat makes up slightly more than 50% of the mass of dry unfermented cocoa bean cotyledons; Forastero beans possess a higher fat content than Criollo beans. The cocoa butter present in the beans is a relatively simple fat, primarily composed of palmitic acid (C16:0, saturated, 25%), stearic acid (C18:0, saturated, 35%), and oleic acid (*cis*-C18:1, monounsaturated, 35%), with small amounts of myristic, linoleic, linolic, and arachidic acids (5%). Stearic acid, the main fatty acid in cocoa butter, has been suggested to be a non-atherogenic type of dietary saturated fat (non-cholesterolemic) because of its lower absorption (Ding et al. 2006).

Cocoa bean quantities of alkaloids vary considerably in ripe seeds of different genetic origins, namely about 0.7%–3.0% of theobromine and 0.1%–0.7%

of caffeine. Depending on the amount of anthocyanins, the polyphenol-storage or pigment cells, which constitute 10%–20% (dry mass) of the cotyledons of cocoa beans, are white to deep purple. Three groups of cocoa bean polyphenols (14%–20% in total) can be distinguished (Wollgast and Anklam 2000b; Ortega et al. 2008): monomeric catechins or flavan 3-ols (ca. 37%, m/m), procyanidins or (epi)catechin oligomers with a degree of polymerization higher than decamers (ca. 58%, m/m), and anthocyanins or glycosylated derivatives of anthocyanidins (ca. 4%, m/m). The main catechin is (–)-epicatechin with up to 35% of the polyphenol content. In smaller amounts, (+)-catechin, as well as traces of (+)-gallocatechin and (–)-epigallocatechin, is found. Beans of the Forastero variety owe their characteristic violet color to the anthocyanin content. White beans of the Criollo type contain approximately two-thirds of the amount of polyphenols found in Forastero beans and do not possess anthocyanins. Both alkaloids and polyphenols are linked to cocoa bitterness. Besides alkaloids and polyphenols, unfermented cocoa beans mainly contain alcohols, aldehydes, organic acids, and esters. It is important to note that plant genetics (cultivar) and environmental conditions of crop cultivation (climate, soil quality and richness, water management, shade canopy management, pollination, etc.) are the first determinants of the final cocoa flavor (Sukha et al. 2008).

## 17.5. Cocoa Bean Fermentation

### 17.5.1. Rationale

Raw cocoa beans have an astringent, unpleasant, and bitter flavor and must be processed after harvest before they can be converted into good-tasting and flavorsome chocolate. Postharvest processing of cocoa beans encompasses curing, grading, and storage, which determine the quality of the final cocoa beans (Lehrian and Patterson 1983; Wood and Lass 1985; Fowler et al. 1998). Curing involves fermentation of the cocoa beans, followed by drying and roasting. Fermentation facilitates removal of the mucilaginous pulp surrounding the beans, as the pulp will otherwise inhibit drying of the beans to a microbiologically stable moisture content, which is necessary for storage and shipping of the beans prior to chocolate manufacture. Also, fermentation lies at the basis of cocoa flavor development. However, it is the cocoa pulp surrounding the beans, and not the cocoa beans themselves, that undergoes microbial fermentation. During this fermentation process, exchange of compounds takes place between the cocoa bean cotyledons and the environment. Actually, cocoa pulp fermentation enables the expression of the flavor potential of the cocoa beans and is hence the second determinant of the final cocoa flavor. Indeed, cocoa pulp fermentation (outside the beans) and endogenous enzymatic conversions (in the beans) play a crucial role in the production of flavor precursors and the degradation of pigments. In principle, the initiation of cocoa bean fermentation corresponds to an incipient germination phase, which is necessary for the hydration of bean components through water uptake by the protein vacuoles within the storage cells and mobilization of the enzymes to prepare the growth of the seedling. Although the further germination phase is undesirable in cocoa beans destined for chocolate production, endogenous enzyme activity is necessary for cocoa flavor development. As there is no evidence to suggest that enzymes from the microorganisms involved in the pulp fermentation process penetrate the beans and create flavor compounds, proper curing of the beans mainly depends on heat and ethanol and acetic acid penetration into the

beans, which are provided during fermentation. This is necessary for inhibiting seed germination by killing the embryo (on the second day of fermentation) and activating hydrolytic enzymes inside the beans for flavor and color development. Also, curing keeps the fat content of the cotyledons at high levels and avoids infection of the beans by leaving the testa closed. Accumulating acetic acid and ethanol in the beans break down the biological barriers (membranes) between the storage and pigment cells within the bean cotyledons, so that various enzymes and substrates are free to mix (Biehl et al. 1982a, 1982b). Ethanol, acids, and water diffusing into the cotyledons act as solvents to transport components to sites of enzyme activity and vice versa, and to enable the subsequent biochemical reactions to produce the flavor and color precursors. The penetrating acetic acid causes the bean pH to drop from an initial value of pH 6.3–6.8 to pH 4.0–4.8, determining the degree of enzymatic activities (see below). Also, the enzyme reaction rates are favored by the increasing temperatures due to microbial activity during fermentation. When oxygen begins to enter the bean, at about 96 h into fermentation, a series of enzymatic, oxidative reactions, which results in a browning of the cotyledon, is initiated. After fermentation, flavor and color are further developed during the drying, roasting, and final processing steps of well-fermented cocoa beans (the third determinant); thus, there is no cocoa and chocolate flavor without fermentation (Rohan 1964; Ziegler and Biehl 1988). Yet the inherited characteristics of the bean set a limit to what can be achieved by fermentation and other processes. In other words, it is impossible to improve genetically inferior material by superior processing technologies. Alternatively, it is quite easy to ruin good-quality cocoa by inadequate curing.

### 17.5.2. Farming Practices

Various cocoa bean fermentation systems have been developed worldwide. The actual methods of fermentation vary in different cocoa-producing countries and regions, and even from one cocoa grower to another within a region or country. The different

fermentation methods are heap (e.g., Ghana and Ivory Coast; Fig. 17.3), box (e.g., Brazil and Malaysia), basket (e.g., Nigeria and Ghana), tray (e.g., Ghana), sack (e.g., Ecuador), and platform fermentation (e.g., Ecuador). Approximately one-half of the cocoa is fermented in some type of box, for many years and worldwide, and the remaining half is fermented by using heaps or other traditional methods that are slowly disappearing. For a detailed description of these methods, see Lehrian and Patterson (1983), Wood and Lass (1985), and Thompson et al. (2007).

Before the start of the fermentation process, the cocoa pods are broken open with machetes; the husk

and placenta of the pods are discarded; and the wet beans are scooped out manually. Usually, infected and black beans are removed and bean agglomerates are separated. Also, mechanical pod breaking and bean extraction occur. When healthy, undamaged pods are opened, the pulp is sterile, although the interior of ripe pods may contain a few hundred microorganisms (yeasts) per gram (Maravalhas 1966; Jespersen et al. 2005). When pulp and beans are removed from the opened pods, the pulp is immediately contaminated with a variety of microorganisms from the environment. Environmental inoculation includes contamination from cocoa pod surfaces (probably the main source); unwashed



**Figure 17.3.** The heap fermentation is the simplest and most commonly used method of fermentation on small farms. It requires the simplest equipment at practically no cost, so that it can be run by a family. In a heap fermentation the wet beans are piled on banana or plantain leaves, which are spread out in a circle on the ground, sometimes raised above soil level to allow easy pulp drainage. When the heap is complete it is covered with more leaves, and these are often held in place by small logs. The cover protects the fermenting mass against surface mold growth and keeps the heat inside. In general, sweatings are allowed to flow away and penetrate into the ground. The size of the heaps varies widely; heaps from about 25 to 2000 kg are common. This method is used throughout West Africa and almost exclusively in Ghana, where farmers are fermenting their beans in heaps of 200 to 500 kg for 6 days with care. The heap fermentation method has been used to produce some of the world's finest standard cocoa available, especially in Ghana.

baskets used for transient seed transport (also an important source); banana and plantain leaves used to construct and cover the heaps; boxes and baskets to retain the heat (to compensate for temperature variations during day and night); and to prevent the surface beans from drying; machetes used for opening of the pods; workers' hands; dried mucilage left on the walls of receptacles from previous fermentations; soil; and insects (Ostovar and Keeney 1973; Jespersen et al. 2005; Camu et al. 2007, 2008a). Hence, cocoa fermentation is a spontaneous fermentation process, to which many, but not all, of the microorganisms contaminating the pulp-bean mass contribute (Schwan and Wheals 2004). Cocoa fermentation takes place batchwise during 2–8 days, depending on the cocoa variety and local practices (fermentation method, methods of drainage and aeration, etc.), with 5–6 days being the most usual.

Criollo beans ferment quickly (2–3 days) and have been reported in the past to have a highly regarded (caramel, nut, or honey), slightly bitter, but usually weak, cocoa flavor. Forastero beans ferment slowly (5–7 days), and their flavor produced on proper processing is stronger (bitter and cocoa flavor). Trinitario beans provide a less intense but sufficient fine taste, which is intermediary between Criollo and Forastero beans. The Arriba-type beans (Nacional) produce cocoa that has a fine flavor, is very aromatic, and has slightly bitter taste.

Besides the type of cocoa beans, critical process parameters for successful fermentation are the healthy status of the cocoa pods; ripeness of the pods; postharvest pod storage; quantity of beans and pulp; bean packing or spreading; fermentation method; batch size; duration of fermentation; mixing and turning of the fermenting mass; seasonal variations and weather conditions; and so on. For instance, storing the harvested cocoa pods for a few days before opening has been considered beneficial for fermentation because the pulp sucrose is already converted into glucose and fructose by cotyledon invertase activity, thus resulting in a faster fermentation (Tomlins et al. 1993; Schwan 1998; Schwan and Wheals 2004). However, the pod ripeness at harvest time remains a crucial factor and appears

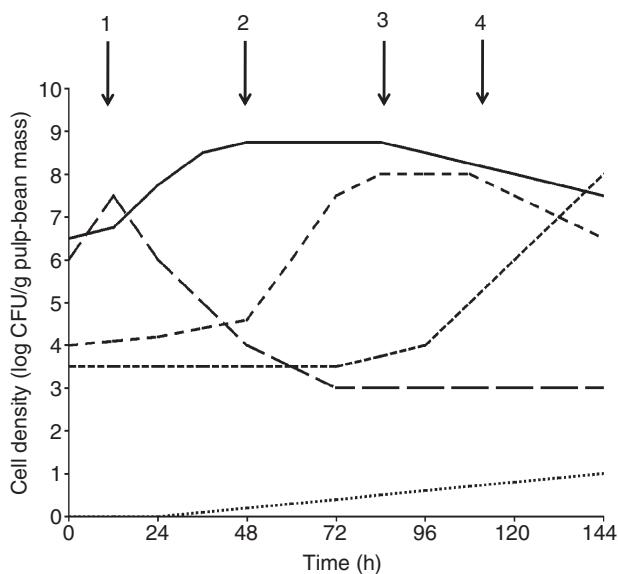
superior to postharvest storage regarding fermented cocoa bean quality (Camu et al. 2008a, 2008b). Also, fresh beans can vary considerably in the ratio of pulp to beans and in the amount of sugars per bean, depending on cocoa cultivar, growing conditions, and postharvest storage of pods. For instance, pulp and beans can contain more water during the wet season, which influences fermentation by affecting aeration of the fermenting mass and thus bean acidity. In such circumstances, up to 20% of pulp of the total fresh pulp-bean mass can be removed, either through a natural “sweatings” drain, with mechanical depulpers, or through addition of pectinolytic enzymes, which enables reduction of the fermentation time from 7 to 4 days, hence lowering the acidity and enhancing the flavor of the final cocoa (Schwan et al. 1995; Schwan and Wheals 2004). For instance, in Malaysia, harvested cocoa pods are stored up to 15 days before breaking and removal of the beans, or the beans are pressed or pre-dried to reduce the pulp volume before fermentation (Lehrian and Patterson 1983). Unfortunately, Malaysian cocoa produced on a large scale is of poor quality because either the beans are not intentionally fermented at all or the fermentation and drying process are not carefully carried out. Typically, beans produced in Brazil and Malaysia present incomplete box fermentation with a low pH (below the recommended value of pH 5.0–5.5), mainly due to low-care practices concerning pod choice and bean pre-processing. To ensure a uniform fermentation, to enhance growth of beneficial microorganisms, and to discourage surface mold growth, fermentations need to be carried out with healthy beans from ripe pods, and heaps and boxes need to be turned every 2 days, which is done practically by forming another heap or by moving the beans from one box or box compartment to another, respectively (Baker et al. 1994). This turning aerates the bean mass and causes the temperature to drop immediately after mixing; the temperature rises again later as more fermentation proceeds. However, turning of small heaps may favor growth of acetic acid bacteria (AAB) that could produce beans with a very high acidity (Camu et al. 2008a). Tray-

fermented cocoa beans do not require turning, as air is allowed to circulate between the trays and sweatings drain away through the bottom of all trays. Trays form a promising alternative to heaps and boxes in cocoa bean fermentation at farm scale. As turning of the fermenting cocoa bean mass seems to be a determining factor for good fermentation practice, fermentation in appropriate bioreactors has been proposed (Schwan and Wheals 2004). Although mechanical turning is not a realistic alternative and forced aeration requires careful control, drum fermentors and stainless steel vessels have been constructed. However, as fermentation of solid material is difficult to control, in particular with respect to homogeneity (gradient formation) and physical (e.g., temperature, pH, dissolved oxygen) and chemical factors (e.g., nutrient availability), new designs of fermentors are needed to have some of these process parameters under control, at least between certain limits (for instance, through turning and cooling), and to speed up and control the pulp-bean mass fermentation process accurately.

## 17.6. Succession of Microorganisms during Cocoa Bean Fermentation

### 17.6.1. The Spontaneous Three-Phase Cocoa Bean Fermentation Process

Successful cocoa bean fermentation requires a succession of microorganisms, or better of microbial activities, reflecting the environmental factors (temperature, pH, and oxygen tension) and the metabolism of substrates available in the cocoa pulp (Ardhana and Fleet 2003; Schwan and Wheals 2004; Camu et al. 2007). Indeed, microbial metabolism is held responsible for the acidity and high temperatures that develop in the fermenting mass; the diffusion of important components in and out of the beans during fermentation; and the enzymatic conversion of constituents in the beans during fermentation and drying (see above). Although overlap often occurs, three main fermentation phases can be considered consecutively (Fig. 17.4): phase 1 or the anaerobic growth of yeasts; phase 2 or the growth of lactic acid bacteria (LAB); and phase 3 or the



**Figure 17.4.** Population dynamics and succession of microbial activities during cocoa bean-pulp mass fermentation. LAB (—); AAB (— —); yeast (— — —); spore-forming bacteria (— · —); and filamentous fungi (· · · · ·). The numbers indicate maximal concentrations of ethanol (1), lactic acid (2), and acetic acid (3), and the maximum temperature (4).



growth of AAB (Schwan et al. 1995; Barel 1998; Ardhana and Fleet 2003; Schwan and Wheals 2004; Camu et al. 2007; Thompson et al. 2007). In general, during fermentation the total count of microorganisms present in the pulp increases during the first 24–36 h and then stabilizes or gradually drops. The initial microbial population is variable in numbers, type, and distribution, depending on cocoa cultivar, pod quality (pod healthiness, ripeness, and storage), and bean pre-processing, and hence the microbial population dynamics vary between fermentation practices, regions, and countries (Camu et al. 2007; Nielsen et al. 2007a).

The microbiology of cocoa bean fermentations carried out in Africa (Ghana, Ivory Coast, Nigeria), Latin America (Belize, Brazil, Dominican Republic, Mexico, Trinidad), and Southeast Asia (Malaysia, Indonesia) has been studied in detail (Ostovar and Keeney 1973; Carr et al. 1979; Passos et al. 1984a, 1984b; Ravelomanana et al. 1985, 1986; Schwan et al. 1995; Ardhana and Fleet 2003; Lagunes-Gálvez et al. 2007; Nielsen et al. 2007a; Thompson et al. 2007; Kostinek et al. 2008). Recent polyphasic studies have dealt with both culture-dependent (encompassing classical microbiological and molecular identification techniques) and culture-independent methods (in particular denaturant gradient gel electrophoresis of PCR-derived 16S rRNA [bacteria]/26S rRNA [yeast] gene amplicons). DNA-based molecular methods have revealed that not only a number of different species of yeasts, LAB, and AAB are important for cocoa bean fermentation, but that different strains within the different species may be involved as well, both often in a certain succession (Jespersen et al. 2005; Camu et al. 2007; Nielsen et al. 2007a).

### 17.6.2. Yeast Fermentation

During the first 24–48 h of fermentation (phase 1), yeasts convert sugars (sucrose, glucose, and/or fructose) into alcohol (ethanol) under conditions of high carbohydrate concentrations (characteristic for fresh cocoa pulp), limited oxygen availability (due to tight packing of the beans), and a pH below 4.0 (due to the high citric acid content in cocoa

pulp). They grow from an initial 2–7 to a maximum 7–9 log cfu/g of cocoa pulp. The remaining sucrose is converted by yeast invertase into glucose and fructose, releasing 18.8 kJ of heat per mole of sucrose that is hydrolyzed. However, as mentioned above, sucrose concentrations depend on pod ripeness and hence bean invertase activity. Furthermore, glucose is preferentially converted into ethanol by anaerobic yeast fermentation, leaving fructose largely unfermented. Pulp ethanol concentrations of approximately 2.5% as a result of yeast fermentation have been reported in the literature. The production of ethanol is accompanied by a moderate temperature increase, as the conversion of sugars into ethanol (and carbon dioxide) is an exothermic process, producing 93.3 kJ per mole of glucose or fructose, elevating the temperature of the fermenting mass from an ambient temperature of 25–30°C to 35–40°C within 48 h. Ethanol disappears upon fermentation due to the oxidation to acetic acid by AAB (see below), oxidative consumption by aerobically growing yeasts upon fermentation, diffusion into the bean cotyledons, sweating, and evaporation. Also, yeasts produce organic acids (e.g., acetic acid, malic acid, and succinic acid), which may have a buffering effect on the cocoa pulp, as well as fusel alcohols, fatty acids, and fatty acid esters, which may contribute to cocoa flavor or, more likely, to precursors of cocoa flavor (Schwan and Wheals 2004). In addition, yeast activity causes reduction of viscosity and drainage of the cocoa pulp due to secretion of pectinolytic enzymes that break down the cell walls in the pulp (Gauthier et al. 1977; Sanchez et al. 1984, 1985; Schwan et al. 1995, 1997; Ardhana and Fleet 2003; Jespersen et al. 2005). The spaces formed between the beans, due to collapse of the parenchyma cells in the pulp, allow air to enter, promoting the growth of LAB and AAB (see below). To speed up the fermentation process and enhance the quality of the final product, pectinases may be added to the pulp, or strains overproducing pectinolytic enzymes may be used to enhance sweating production (Schwan and Wheals 2004).

Several studies have revealed the involvement of an abundant yeast diversity, encompassing species of *Candida* (including former *Torulopsis*),

*Cryptococcus*, *Hanseniaspora* (and their anamorphs *Kloeckera*), *Kluyveromyces* (including *Lachancea thermotolerans*, formerly *Debaromyces thermotolerans*), *Meyerozyma* (former *Pichia*), *Millerozyma* (former *Pichia*), *Pichia* (including former *Issatchenkia* and *Hansenula*), *Rhodotorula*, *Saccharomyces* (including *Kazachstania exigua*), *Saccharomycopsis*, *Schizosaccharomyces*, *Torulaspora*, and *Wickerhamomyces* (including former *Pichia*) (Schwan et al. 1995; Fowler et al. 1998; Ardhana and Fleet 2003; Schwan and Wheals 2004; Jespersen et al. 2005; Lagunes-Gálvez et al. 2007; Nielsen et al. 2007a; Thompson et al. 2007; Daniel et al. 2009). The most frequently mentioned yeast species are *Saccharomyces cerevisiae* (synonym *Saccharomyces chevalieri*), *Hanseniaspora guilliermondii* (anamorph *Kloeckera apsis*), *Pichia kudriavzevii* (formerly *Issatchenkia orientalis*, anamorph *Candida krusei*), *Pichia membranifaciens*, *Pichia kluyveri*, *Pichia fermentans*, and *Pichia anomala* (Schwan et al. 1995; Ardhana and Fleet 2003; Schwan and Wheals 2004; Jespersen et al. 2005; Nielsen et al. 2005, 2007a; Lagunes-Gálvez et al. 2007; Daniel et al. 2009). Thanks to an accurate identification, based on a combination of molecular analyses and morphological and physiological observations, Daniel et al. (2009) have identified *P. kudriavzevii*, *S. cerevisiae*, and *Hanseniaspora opuntiae* as the main yeast species involved in Ghanaian cocoa bean heap fermentations, confirming earlier studies (Jespersen et al. 2005; Nielsen et al. 2005, 2007a), and further reported on several species that have not been found previously in cocoa bean fermentation. Culture-independent investigations have shown the dominant role of *H. guilliermondii/opuntiae*, *P. kudriavzevii*, and *P. membranifaciens* in Ghanaian heap fermentations, whereas *S. cerevisiae* has been found almost exclusively in tray fermentations (Nielsen et al. 2005, 2007a). Based on denaturing gradient gel electrophoresis (DGGE) profiles, Leal et al. (2008) have detected *Candida tropicalis*, *Candida pseudotropicalis*, *Kluyveromyces marxianus*, and *S. cerevisiae* in plastic basket fermentations carried out in Brazil during the first 48 h. In the study of Daniel et al. (2009), strains of the three main species and

*P. kluyveri* var. *kluyveri* are represented by multiple ones, as minor sequence variation in rRNA gene cluster sequences and the actin (*ACT1*) gene among different isolates was observed. In contrast, in the case of the dominant yeast species *P. kudriavzevii*, *S. cerevisiae*, *P. membranifaciens*, *P. kluyveri*, and *H. guilliermondii*, in the study of Jespersen et al. (2005), strain variation was examined by chromosome length polymorphism, indicating the presence of a considerable diversity of individual strains in fermentations, on the pod surfaces, and at different production sites. Species solely isolated during single fermentations, at the start of the fermentations, and from the associated materials might be considered members of the surrounding ecosystems that occasionally become established during fermentation, if their physiological properties favor growth under the fermentation conditions in place. This holds true for bacteria as well (see below).

In general, *S. cerevisiae*, potentially due to its rapid growth, pectinolytic activity, and ethanol tolerance, is the most often detected species and also the most abundant during cocoa bean fermentation, usually after an initial fermentation phase dominated frequently by *H. guilliermondii/opuntiae* (Schwan et al. 1995; Ardhana and Fleet 2003; Jespersen et al. 2005; Daniel et al. 2009). For instance, Ardhana and Fleet (2003) have shown that *H. guilliermondii* grows rapidly at low pH during the first 24–36 h of fermentation, followed by more ethanol- and heat-tolerant *S. cerevisiae* and *C. tropicalis* at 48–120 h during wooden-box fermentation of cocoa beans in East Java. Also, during the initial phase of cocoa bean heap fermentations in Ghana, it has been shown that *H. guilliermondii/opuntiae* plays an important role (Jespersen et al. 2005; Nielsen et al. 2005, 2007a; Daniel et al. 2009), as is the case for box fermentations in the Dominican Republic (Lagunes-Gálvez et al. 2007). Upon prolonged fermentation, the more ethanol-tolerant yeast species, namely *S. cerevisiae* (tray), *P. kudriavzevii* (heap), and *P. membranifaciens* (tray and heap), become predominant in Ghanaian fermentations. This yeast distribution is reflected in the citrate fermentation capacity of yeasts and LAB and their coexistence in the beginning of the cocoa bean

fermentation process (Daniel et al. 2009). Whereas LAB are responsible for a fast citrate consumption at the start of the fermentation (see below), *H. opuntiae* is not able to assimilate citrate and grows, together with the other yeasts, on sucrose as an energy source. The tolerance of low pH values by *H. opuntiae* is in accordance with environmental conditions prevailing during initial cocoa bean fermentation (low pH at the start of the fermentation due to a high citrate concentration), *S. cerevisiae* being the most sensitive yeast species toward the lowest pH values and hence proliferating after *H. opuntiae* (Daniel et al. 2009). Higher temperatures and ethanol concentrations toward the end of a cocoa bean fermentation process, due to increased microbial activity, influence survival of various cocoa-specific yeast species, as their tolerance toward these external factors are variable (Ardhana and Fleet 2003; Jespersen et al. 2005; Nielsen et al. 2005, 2007a; Daniel et al. 2009). The limited number of yeasts capable of growing at 45°C and higher explains the disappearance of the yeast population once ethanol oxidation by AAB, which causes a substantial temperature increase during cocoa bean fermentation, has started and the appropriate energy resources have been exhausted, resulting in a higher pH and greater aeration (Schwan and Wheals 2004; Camu et al. 2007; Daniel et al. 2009).

### 17.6.3. LAB Fermentation

Continuing yeast activity causes further ethanol production and more pulp draining away, allowing more air ingress, while both temperature and pH of the fermenting mass increase up to 40°C and pH 4.0, respectively. These conditions are favorable for the growth of bacteria. During phase 2, around 24–72 h into fermentation, the microaerophilic and aciduric LAB present from the start of the fermentation grow rapidly, from an initial 3–5 to a maximum 7–9 log cfu/g of cocoa pulp. This coincides with a rapid decline in the dominance of the yeast population. LAB ferment a wide range of sugars (mainly glucose and fructose) and convert some organic acids (for instance, citric acid and malic acid) into lactic acid,

the main fermentation end-product and, depending on the type of LAB strain, acetic acid, ethanol, and carbon dioxide. However, most LAB species prefer glucose as energy source, while some LAB species use fructose as an alternative external electron acceptor and convert it into mannitol (Camu et al. 2007, 2008a). The relative proportion of all these fermentation end-products will change the composition of the pulp and consequently may influence microbial succession. The lactic acid concentration peaks at around 0.5%.

Earlier and classical microbiological studies have revealed a dominance of various species of *Lactobacillus* compared with species of *Leuconostoc*, *Pediococcus*, and/or *Lactococcus* (Ostovar and Keeney 1973; Carr et al. 1979; Passos et al. 1984a, 1984b; Thompson et al. 1997, 2007; Nielsen et al. 2007a; Table 17.1). In general, it appeared that heterofermentative LAB occur in higher numbers than homofermentative LAB, and *Lactobacillus* spp. are present in the early stages of fermentation, whereas *Lactococcus* spp. occur during the final stages (Schwan et al. 1995; Lagunez-Gálvez et al. 2007; Nielsen et al. 2007a). West African cocoa bean heap fermentations have been studied most extensively, encompassing both culture-dependent and culture-independent methodologies. In general, through classical microbiological analysis, the main LAB species described are *Lactobacillus brevis*, *Lactobacillus fermentum*, and *Lactobacillus plantarum* (Carr et al. 1979; Nielsen et al. 2007a; Kostinek et al. 2008). Recently, it has been shown by both culture-dependent and culture-independent methods that *Lactobacillus plantarum* and *Leuconostoc pseudomesenteroides* dominate at the onset of Ghanaian cocoa bean heap fermentation and *Lactobacillus fermentum* toward the end, while *Fructobacillus pseudoficulneus* (formerly *Leuconostoc pseudoficulneum*; this species was not found through DGGE monitoring) and *Weissella ghanensis* (only in one heap fermentation out of seven heaps studied) constitute a small part of the LAB community, whether or not as opportunistic species (Camu et al. 2007; Nielsen et al. 2007a). These investigations indicate a rather restricted LAB diversity. Other LAB species have been found,

**Table 17.1.** Diversity of lactic acid bacteria associated with cocoa bean fermentations in different countries (original species names are used).

Country	Fermentation method	Microorganisms found	Reference
Trinidad	Box	<i>L. lactis</i> ; <i>Lact. fermentum/plantarum</i> ; <i>Leuc. mesenteroides</i>	Ostovar and Keeney (1973)
Ghana	Box	<i>Lact. collinoides/fermentum/mali/plantarum</i>	Carr et al. (1979)
Ghana	Heap and tray	<i>L. lactis</i> (heap only); <i>Lact. brevis/fermentum/ghanensis</i> <sup>a</sup> (heap only)/ <i>hilgardii</i> (heap only)/ <i>plantarum/rossii</i> (heap only); <i>Leuc. pseudoficulneum/pseudomesenteroides</i> ; <i>Ped. acidilactici</i> (culture-dependent analysis) <i>Lact. fermentum/plantarum</i> ; <i>Ped. acidilactici</i> ; <i>Leuc. pseudoficulneum/pseudomesenteroides</i> (culture-independent analysis)	Nielsen et al. (2007a)
Ghana	Heap	<i>Ent. casseliflavus</i> ; <i>Enterococcus</i> spp.; <i>L. lactis</i> subsp. <i>Lactis</i> ; <i>Lact. brevis/cacaonum</i> <sup>b</sup> / <i>fabifermentans</i> <sup>b</sup> / <i>fermentum/plantarum</i> ; <i>W. fabaria</i> <sup>c</sup> / <i>ghanensis</i> <sup>d</sup> / <i>paramesenteroides</i> , <i>Weissella</i> spp. (culture-dependent analysis)	Camu et al. (2007, 2008a)
Malaysia	Box	<i>Lact. collinoides/plantarum</i>	Carr et al. (1979)
Brazil	Box	<i>L. lactis</i> ; <i>Lact. acidophilus/brevis/casei/delbrueckii/lactis/plantarum</i> ; <i>Leuc. mesenteroides</i> ; <i>Ped. acidilactici/dextrinicus</i>	Passos et al. (1984b)
Belize	Box	<i>Lact. brevis/buchneri/casei/casei</i> subsp. <i>pseudopantarum/gasserii/cellobiosus/delbrueckii/fermentum/fructivorans/kandleri/plantarum</i> ; <i>Leuc. mesenteroides/oenosis/paramesenteroides</i>	Thompson et al. (1997)
Indonesia	Box	<i>Lact. cellobiosus/hilgardii/plantarum</i>	Ardhana and Fleet (2003)
Dominican Republic	Box	<i>Lact. brevis/pentosus/paracasei</i> subsp. <i>paracasei(para) plantarum</i>	Lagunes-Gálvez et al. (2007)
Nigeria	Heap and tray	<i>Enterococcus</i> spp. (tray only); <i>Lact. brevis/fermentum/plantarum</i> ; <i>Ped. acidilactici</i>	Kostinek et al. (2008)

<sup>a</sup>Nielsen et al. (2007b).<sup>b</sup>De Bruyne et al. (2009a).<sup>c</sup>De Bruyne et al. (2009b).<sup>d</sup>De Bruyne et al. (2008).

but they have often been isolated as single strains or identified as rare bands in DGGE profiles, which rather reflects opportunistic contamination from the environment (Camu et al. 2007; Nielsen et al. 2007a). Using classical microbiological analysis, Ardhana and Fleet (2003) showed that *Lactobacillus cellobiosus* (later synonym of *Lact. fermentum*) and *Lact. plantarum* are the dominant LAB involved in Indonesian box fermentations. Whereas *Lact. plantarum* was especially significant during the first 24 h

of fermentation, *Lact. cellobiosus* dominated 36–48 h, and *Lact. hilgardii* died off by 48 h, into fermentation. Heap and tray fermentations carried out in Nigeria are characterized by an increasing *Lact. plantarum* population in the first half of fermentation, and an increasing *Lact. fermentum/Lact. brevis* population (tray) and decreasing *Ped. acidilactici* (heap and tray) and *Lact. fermentum/Lact. brevis* population (heap) in the second half of the fermentation (Kostinek et al. 2008). Taken together, these

data indicate that *Lact. plantarum* and *Lact. fermentum* seem to be indigenous to the fermentation of cocoa beans worldwide. Moreover, *Lact. fermentum* seems to play a significant role (see below).

Although a few yeasts assimilate citric acid (Jespersen et al. 2005; Daniel et al. 2009), LAB species are responsible for the breakdown of citric acid during cocoa bean fermentation. Citric acid is converted via oxaloacetate into acetate and pyruvic acid, the latter, in turn, being converted into end-products of pyruvate metabolism (lactic acid, acetic acid, acetaldehyde, ethanol, diacetyl, etc.; Camu et al. 2007, 2008a). Consumption of citrate by *Leuc. pseudomesenteroides* (heterofermentative), *Lact. plantarum* (homofermentative), and *Lact. fermentum* (heterofermentative) at the early stages of Ghanaian cocoa bean heap fermentation, under low pH conditions, explains the importance of these bacteria during this stage of the fermentation process, thereby avoiding competition with the depectinizing (citrate-negative) yeasts, which meanwhile degrade sugars to ethanol anaerobically. Dominance of specific strains of *Lact. fermentum* throughout cocoa bean fermentation, usually in succession of *Lact. plantarum* that dominates at the beginning of the fermentation, can be ascribed to their competitive metabolism (efficient use of glucose and fructose as energy source and external electron acceptor, respectively), their acid and ethanol tolerance (lower than for *Lact. plantarum*), and their higher oxygen tolerance than *Lact. plantarum* (Camu et al. 2007, 2008a). Assimilation of citric acid causes the pH of the cocoa pulp to increase from pH 3.5 to 4.2–5.0, allowing the growth of other bacteria (Camu et al. 2007, 2008a). Another important feature of *Lact. fermentum* growth is the production of mannitol out of fructose, allowing more acetic acid to be produced, which takes place simultaneously with the conversion of citric acid via pyruvate into acetic acid, from a physiological point of view to regenerate the NAD<sup>+</sup> cofactor and produce extra ATP, explaining good growth of *Lact. fermentum* strains at low pH (Camu et al. 2007, 2008a). Moreover, metabolic connections exist between citrate fermentation and processes such as amino acid interconversion and mannitol production. As is the case for the

yeasts, LAB numbers decline during later stages of fermentation, mainly due to exhaustion of energy sources, very high ethanol concentrations, and very high temperatures. To conclude, LAB do play an important role, although thought differently in the past, during the cocoa bean fermentation process (Table 17.2).

#### 17.6.4. AAB Fermentation

During phase 3, from 24 to 112 h into fermentation, aerobic AAB, which occur from the very early stages of fermentation and hence survive the initial steps of this process, persist until the end when the conditions for AAB growth are optimal. When more of the pulp is metabolized and drained away, aeration increases, and when the temperature rises above 37°C, thermotolerant AAB grow from an initial 3–5 to a maximum 5–8 log cfu/g of cocoa pulp. Furthermore, turning of large heaps and moving of the beans during box fermentation favor AAB growth (cell counts, acetic acid concentration) and increase the maximum fermentation temperature (Carr et al. 1979; Nielsen et al. 2007a; Camu et al. 2008a). Concomitantly, turning of a small heap results in the production of too much acetic acid, resulting in fermented dry cocoa beans with an elevated level of acetic acid, which in turn results in a sour-tasting chocolate (Camu et al. 2008a, 2008b).

The main activity of AAB is the oxidation of ethanol, initially formed by the yeasts, into acetic acid, an enzymatic reaction that provides AAB with the necessary energy. This exothermic reaction, which produces 496 kJ per mole of ethanol that is oxidized, is mainly responsible for the rise in temperature of the fermenting mass, which can reach 45–50°C or higher. The decline in the ethanol concentration coincides with a decline in the lactic acid concentration, indicating simultaneous oxidation by AAB of ethanol to acetic acid and of lactic acid to carbon dioxide and water. The acetic acid concentration peaks at around 2% and then declines probably due to evaporation at the high temperature of the fermenting mass and further metabolism (Camu et al. 2007, 2008a, 2008b; Nielsen et al. 2007a). Indeed, some AAB are responsible for the overoxi-



**Table 17.2.** Functional role of lactic acid bacteria associated with cocoa bean fermentation.

Functional property	Effect on cocoa bean fermentation
Consumption of citric acid	Causes a rapid start up of the fermentation under low pH conditions, avoiding competition with sucrose- (glucose-)consuming, citrate-negative, depectinizing yeasts Causes a pH increase (replacement of citric acid by lactic acid and acetic acid), in turn controlling bacterial growth Stimulates amino acid conversions
Production of lactic acid	Plays a pH regulatory role Controls microbial contamination from the environment Beneficial for the growth of AAB (and yeasts)
Production of mannitol	Favors production of acetic acid (volatile) instead of lactic acid (nonvolatile) out of fructose Beneficial for the growth of AAB
Production of flavor precursors (acetate, pyruvate catabolites, and amino acid conversion products)	Provides the necessary precursors for further flavor and color development during secondary processing
Create appropriate conditions for proteolysis, flavor precursor formation, and color development within the beans	Controls flavor (precursor) and color development in the beans

dation of acetic acid into carbon dioxide and water, in turn liberating 1754 kJ per mole of acetic acid that is overoxidized. Also, oxidation of lactic acid and acetic acid results in a further pH increase.

As for the LAB species, AAB diversity associated with cocoa bean fermentation is restricted. Based on classical microbiological analysis, the AAB species involved are *Acetobacter aceti*, “*Acetobacter roseum*,” and “*Acetobacter suboxydans*” in Trinidadian box fermentations (Ostovar and Keeney 1973); “*Acetobacter ascendens*,” *Acetobacter rancens* (later synonym of *Acetobacter pasteurianus*), *Acetobacter xylinum* (now *Gluconacetobacter xylinus* subsp. *xylinus*), and *Gluconobacter oxydans* in Ghanaian heap fermentations (Carr et al. 1979); *Acetobacter lovaniensis*, *A. rancens*, *A. xylinum*, and *G. oxydans* in Malaysian box fermentations (Carr et al. 1979); *A. aceti* subsp. *liquefaciens* (now *Gluconacetobacter liquefaciens*), *A. pasteurianus*, *Acetobacter peroxydans*, “*G. oxydans* subsp. *suboxydans*” (now *G. oxydans*) in Brazilian box fermentations (Passos and Passos 1985); and *Acetobacter* spp. and *G. oxydans* in Belizean box fermentations (Thompson et al. 1997). In general, members of the

genus *Acetobacter* are found more frequently than those of *Gluconobacter*. Molecular analysis reveals that *A. pasteurianus* (first half of the fermentations), *Acetobacter syzygii* (first half), *Acetobacter tropicalis* (later stages of fermentation), and occasionally *Acetobacter malorum* and *G. oxydans*, are the dominating AAB during Ghanaian cocoa bean heap and tray fermentations (Nielsen et al. 2007a). *Acetobacter lovaniensis* is the only species identified out of five isolates during a box fermentation carried out in the Dominican Republic (Lagunes-Gálvez et al. 2007). Camu et al. (2007, 2008a) have found *Acetobacter fabarum* (Cleenwerck et al. 2008), *Acetobacter ghanensis* (Cleenwerck et al. 2007), *A. pasteurianus*, and *Acetobacter senegalensis* during Ghanaian cocoa bean heap fermentations, among which *A. pasteurianus* dominates. The predominance of specific strains of *Acetobacter (pasteurianus)* over *Gluconobacter* can be ascribed to its preference for oxidation of ethanol, which is readily available in the pulp-bean mass, instead of glucose fermentation; its growth on mannitol and lactate; and its tolerance to heat and ethanol (Ardhana and Fleet 2003; Camu et al. 2007; Nielsen et al. 2007a). Isolation of

*Gluconobacter* may indicate poor fermentation of the pulp-bean mass, as glucose fermentation will dominate ethanol oxidation, resulting in the production of organic acids other than acetic acid or other fermentation end-products by other bacteria. Finally, exhaustion of ethanol and an increase in the temperature of the fermenting pulp-bean mass cause the death of the AAB, halting the whole fermentation process.

#### 17.6.5. *Bacillus* Growth

In the later stages of the cocoa bean fermentation process, as sugar levels further decrease and pH, temperature and aeration increase, aerobic spore-forming bacteria of the genus *Bacillus* (*B. cereus*, *B. licheniformis*, *B. megaterium*, *B. pumilus*, *B. sphaericus*, *B. subtilis*) may dominate. This population varies from 4 log cfu/g of pulp during the first 3 days of fermentation to 7–9 log cfu/g of pulp at the end of fermentation (Ostovar and Keeney 1973; Carr et al. 1979; Lehrian and Patterson 1983; Schwan et al. 1986, 1995; Ardhana and Fleet 2003; Nielsen et al. 2007a). The exact role of *Bacillus* spp. during cocoa bean fermentation has not been fully elucidated. These bacteria are able to produce a variety of metabolic end-products, relative to fermentation conditions, that contribute to acidity (lactic acid, acetic acid), desirable flavors (acetoin, 2,3-butanediol, tetramethylpyrazine), and typical hammy off-flavors (C3 to C5 free fatty acids) of cured cocoa beans (Schwan et al. 1986; Schwan and Wheals 2004). Alternatively, *Bacillus* spp. may contribute to pectinolysis, as a high proportion of pectinolytic strains among cocoa isolates have been found, in particular after yeast decline upon fermentation (Ouattara et al. 2008). In this context, it can be mentioned that species of *Aeromonas*, *Erwinia*, and *Pantoea*, which have been shown by several investigators to occur during cocoa bean fermentation, may contribute to pectinolysis too (Camu et al. 2008a).

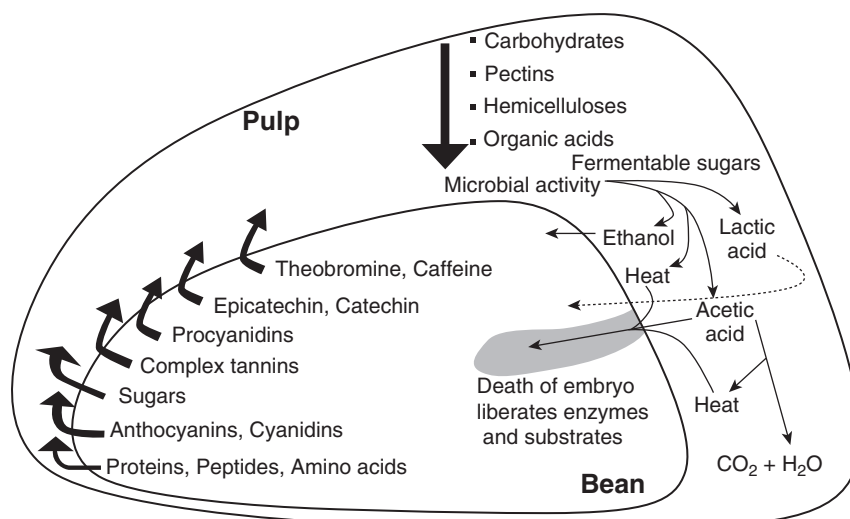
#### 17.6.6. Mold Growth

Filamentous fungi are normally found at the surface and in the cooler and more aerated parts of the pulp-

bean fermenting mass and during the drying process (Maravalhas 1966; Lehrian and Patterson 1983). During fermentation, steps are taken to suppress the growth of these fungi, because their metabolic end-products may lead to a bad cocoa flavor (Ribeiro et al. 1986). Internal molding of the beans, a moldy odor of the beans, and increased free fatty acid levels are serious commercial defects. Factors affecting bean integrity (unhealthy or damaged cocoa pods, poor handling, and deferred processing) may increase fungal contamination (Mounjouenpou et al. 2008). Moldy cocoa beans may contain species of *Absidia*, *Aspergillus*, *Fusarium*, *Mucor*, *Penicillium*, *Rhizopus*, and *Trichoderma* (Maravalhas 1966; Niles 1981; Guehi et al. 2007a, 2007b; Mounjouenpou et al. 2008). Furthermore, it is likely that excessive mold growth during the later stages of fermentation will enhance further mold growth and potential mycotoxin production during the subsequent drying phase (Mounjouenpou et al. 2008). Alternatively, Ardhana and Fleet (2003) have suggested that pectinolytic filamentous fungi may play a role in the degradation of cocoa pulp pectin, as they display strong pectinolytic activity. In addition, the effect of filamentous fungi on bean composition with respect to starch, proteins, and lipids, which could contribute significantly to the final quality of cocoa beans, has been speculated on.

### 17.7. Biochemical Changes in the Cocoa Beans during Fermentation and Drying

Physicochemical processes within the beans encompass diffusion, internal damage (due to acetic acid penetration), and enzymatic processes (Fig. 17.5), which are influenced by several environmental factors, such as pH (determined by the duration and intensity of acidification), temperature (determined by the speed of fermentation), and moisture (determined by the speed of fermentation and drying) (Schwan et al. 1995; Thompson et al. 2007). Biochemical and physical changes within the bean cotyledons can be divided into two broad phases. The first phase is the anaerobic hydrolytic phase, which occurs during the first 3–4 days of fermentation. It involves absorption of water and diffusion



**Figure 17.5.** Biochemical changes and diffusion processes in pulp and beans during cocoa bean fermentation (after Lopez 1986).

of substrates in the bean tissue during fermentation after seed death occurred. This allows hydrolysis of bean cotyledon proteins (by proteases), sucrose (by invertases), and anthocyanins (by glycosidases) (Voigt et al. 1994a, 1994b; Voigt and Biehl 1995; Amin et al. 1998; Hansen et al. 1998; Puziah et al. 1998a, 1998b; Lercetau et al. 1999; Laloi et al. 2002; Schwan and Wheals 2004; Afoakwa et al. 2008; Jinap et al. 2008). The second phase is the aerobic oxidative phase, during which penetration of oxygen through the testa causes oxidative changes and complexation of polyphenols and protein material at the surface of the cotyledon from the fourth day onwards. The enzymatic reactions are of short duration, as optimal conditions of pH (decreasing in the beans) and temperature (increasing in the beans) are transient during fermentation. Some enzymes are strongly inactivated (aminopeptidase, invertase, and polyphenol oxidase) or partly inactivated (carboxypeptidase) during fermentation, except for aspartic endoprotease and glycosidases, which remain active during the whole fermentation process. For instance, if the bean cotyledon pH becomes too acid too soon ( $\text{pH} < 4.5$ ), there will be both a final reduction in flavor precursors and an overacid final product. Also, a certain amount of

moisture (increasing during fermentation and decreasing during drying) is necessary to allow enzymes and their substrates to react to form the desirable products. Further, moisture contributes to the diffusion of ethanol, acetic acid, and, to a lesser extent, lactic acid within the beans, and polyphenols and alkaloids outward the beans. Browning reactions (enzymatic and nonenzymatic) at the surface of the cotyledons, which are manifested toward the end of the fermentation (aerobic phase) and are, to some extent, responsible for inhibiting enzyme activities, cause tanning of proteins and removal of astringency and bitterness. Also, the ability of tannin formation results in the reduction of off-flavors associated with the roasting of peptide and protein material. Finally, the oxidative phase contributes to the formation of ancillary (e.g., fruity) flavors. Consequently, there is a need for a balance between the length of fermentation, environmental factors, and microbial activities that influence enzymatic activities within the bean cotyledons. Moreover, the heterogeneity of (large) heaps and slight, local differences in microbial numbers and sugars available for fermentation may determine flavor precursor formation within the beans. This may result in slight flavor differences characterizing the resulting

chocolate products (Camu et al. 2008a, 2008b). Controlled fermentation and addition of exogenous enzymes may contribute to steered flavor precursor formation (Yusep et al. 2002; Camu et al. 2008b).

Apparently, there is no change in starch and fat content during fermentation and drying (Lehrian and Patterson 1983). However, as the nonfat portion of the cocoa beans is the source of polyphenols, the polyphenol content and antioxidant properties of chocolate products is determined by polyphenol levels in the cocoa solids of these products. Therefore, the flavor-enhancing properties of cocoa bean fermentation must be balanced against the polyphenol health effects, when polyphenol-enriched chocolate products are to be produced. The potential availability of cocoa beans with various degrees of fermentation provides the chocolate manufacturer with the opportunity to produce chocolate products that may meet the diverse needs of the modern consumer (Thompson et al. 2007).

### 17.8. Optimal Fermentation Course and End of Fermentation

The cocoa bean fermentation process lies at the basis of the entire chocolate-making process. An optimal fermentation course requires appropriate microbial population dynamics, that is, a correct succession of microorganisms and concomitant activities, which are at the basis for development of flavor precursors within the beans and characteristic coloration of the beans. Therefore, an optimal internal pH of the beans and a normal temperature increase during fermentation have to be established. The minimum pH that gives acceptable cocoa mass is 5.2; however, the actual pH may be slightly lower, which is related with acidity development during fermentation. The temperature increase reflects the actual functioning of the microorganisms. The rate of temperature increase is a quantitative measure for the type of fermentation (alcoholic, lactic, and/or acetic) as it is correlated with the energy released and hence may be correlated with the eventual cocoa quality. Barel (1998) considers a rise in temperature to around 45°C during the first 48 h of fermentation as satisfactory. This temperature increase is,

however, mainly ascribed to the action of AAB. Therefore, according to Barel (1998), LAB are less desirable or not wanted at all during cocoa bean fermentation. If LAB constitute a high percentage of the total microbial population during cocoa bean fermentation, high concentrations of lactic acid will be produced, as the breakdown of carbohydrates then contributes mainly to total acidity (more lactic acid formation by LAB and less ethanol production by yeasts) of the cocoa beans. Since lactic acid is not volatile, it will remain in the chocolate after manufacturing, producing excessive sourness that masks the chocolate flavor (Holm et al. 1993; Jinap 1994; Jinap and Zeslinda 1995). However, as described above, the functional role of LAB in developing well-fermented cocoa beans has been underestimated, and a noncompetitive cooperation between yeasts and LAB exists in the fermenting mass (Camu et al. 2007).

As cocoa beans vary in their degree of fermentation, because of the unpredictable fermentation step due to its spontaneous nature, a fermentation index has been introduced as a quantitative measure for the degree of cocoa bean fermentation (Shamsuddin and Dimick 1986). Cocoa beans can be underfermented (too short fermentation), overfermented (too long fermentation), or optimally fermented (exact fermentation time), with the concomitant sensory attributes of cocoa beans and chocolate made from these cocoa beans. Traditionally, the cut test is performed additionally (del Boca 1962). It indicates the quality of the fermented beans, mainly based on the intensity of color (gray, purple, or brown) and formation of a cracked interior.

A key decision to obtain high-quality fermented cocoa beans is to remove the beans from their fermentation environment to start the drying process. As mentioned above, when all of the ethanol is oxidized into acetic acid and then further overoxidized into carbon dioxide and water, the fermentation stops, as energy sources for microbial growth are no longer available and temperature increases too high to allow survival of the desirable microorganisms. Extending the fermentation can result in the production of off-flavors and putrefaction by undesirable microbes. The following factors may

collectively indicate when fermentation is optimal: (1) decrease in the temperature of the fermenting mass; (2) smell of the fermenting mass (distinct smell of alcohol and acetic acid during the early and late stages of cocoa bean fermentation, respectively); (3) visual appearance of molds at the surface of the fermenting mass; (4) plumping or swelling of the beans (the water content of fermenting beans increases from approximately 35% at harvest time to approximately 40% after bean death); (5) external color of the beans; (6) internal color of the beans as observed in the bean cut test; and (7) internal pH of the beans. However, other factors may influence this decision, in particular when certain components of the cocoa beans have to be valorized in functional food products (see below).

## 17.9. Further Processing of Fermented Cocoa Beans

### 17.9.1. Drying of Fermented Cocoa Beans

After 6 days of fermentation, the drying process of the fermented cocoa beans can start. At this stage, the fermentation must be stopped completely; the moisture content of the beans must be reduced from an initial 40%–60% to 6%–8% to avoid growth of molds within the beans during storage; and the major part of acetic acid formed during fermentation must be eliminated (Thompson et al. 2007). The drying process relies on air movement to remove water. Both sun drying (5–10 days) and mechanical drying (maximum 60°C for at least 48 h) are applied. Uniform drying is ensured by mixing the beans regularly. Water removal results in a pronounced reduction in the number of viable microorganisms (Schwan and Wheals 2004). Furthermore, biochemical processes that are important for the flavor and color development of cocoa beans, initiated during fermentation, continue during drying. A slow migration of moisture throughout the bean will transport flavor precursors that were formed during fermentation. Hence, some oxidation will occur and some excess acids, in particular acetic acid, may volatilize through the shell, phenomena that are both beneficial. Also, nonvolatile lactic

acid is partly transported by the water from the bean to the shell (Wood and Lass 1985; Nganhou et al. 2003; Thompson et al. 2007). Finally, rising temperatures and insufficient moisture become inhibiting factors of enzymatic activity. Also, the efficiency of the drying process will determine the shelf life of the cured product. Dry beans weigh on average 1 g.

### 17.9.2. Roasting of Dried Fermented Cocoa Beans

Roasting (at 100–150°C for 45–70 min) is the most important technological operation in the processing of dried fermented cocoa beans, as it produces the characteristic “cocoa flavor” out of flavor precursors, that is, free amino acids, peptides, and reducing sugars formed during fermentation and drying through non-enzymatic browning reactions and Strecker degradation and/or thermal degradation (Ziegler and Biehl 1988; de Brito et al. 2000; Granvogl et al. 2006; Beckett 2009). It brings about the formation of the characteristic brown color, mild aroma, and texture of roasted beans. Concomitantly, the moisture content of the beans decreases to 2% and acetic acid is further liberated. Roasting conditions, mainly temperature and time, affect the properties of roasted beans, such as concentration of volatile flavor compounds, total acidity, polyphenols, and fat content. Pyrazines may be produced during fermentation, due to growth of bacilli (see above), but they are mainly formed during roasting; tetramethylpyrazines (milk coffee, mocha, roasted, green), together with 2,5-dimethylpyrazines (green, ether, rum), have been suggested as indicators of the roasting of cocoa beans (Chaveron et al. 1989; Jinap et al. 1998, 2008; Yusep et al. 2002). Other typical cocoa flavor compounds include aldehydes, pyroles, furans, alcohols, ketones, organic acids, and esters (Ziegler and Biehl 1988; Ziegler 1991; Jinap et al. 1995; Puziah et al. 1998a, 1998b; Frauendorfer and Schieberle 2006, 2008; Beckett 2009). Whereas the odor-active volatiles, acetic acid (sour, vinegar-like note), 2-phenylethanol (flowery), 2- and 3-methylbutanoic acid (buttery, rancid, sweaty), 3-methylbutanal (malty, chocolate), and ethyl



2-methylbutanoate (fruity), are already formed during fermentation, others such as 3-methylbutanal, phenylacetaldehyde (sweet, honey-like), and 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone (caramel-like, sweet) as the most important ones are intensified or formed during roasting and probably further steps in cocoa processing, depending on the chocolate recipe (Frauendorfer and Schieberle 2006, 2008; Afoakwa et al. 2008; Beckett 2009). Direct relationships are found between the initial composition of cocoa beans (depending on genotype and growth conditions of the crop), postharvest processing (pod storage, pulp preparation, fermentation, and drying), and subsequent processing (roasting, alkalization, and conching) and their effects on flavor formation and final flavor character in chocolate (Afoakwa et al. 2008).

### *17.9.3. Impact of Cocoa Processing on the Nutritional, Organoleptic, and Health Status of Chocolate*

Processing (conditions) affect the composition and properties of the final chocolate, not only with respect to, for instance texture, color, and mouthfeel, but also concerning flavor intensity, nutritional composition, and content of health-promoting compounds. An example is the desirable balance between a bitter taste and a healthy proportion of anti-oxidative polyphenols. Indeed, starting from the unfermented beans, there is a polyphenol loss of about 85% following processing (Wollgast and Anklam 2000b; Misnawi et al. 2004). Similarly, losses of other desirable health-promoting constituents may occur. Therefore, precautions have to be taken to guarantee a certain level of such compounds in the final product.

## **17.10. Use of Starter Cultures for Fermentation of Cocoa**

### *17.10.1. Rationale*

Technological challenges at the level of natural cocoa bean fermentation may have an impact on the quality of the cocoa beans and hence on the quality

of the cocoa and chocolate made from cocoa beans. A possible impact may be expected not only on the color and flavor development of the beans during fermentation, but also on sugar and fat contents, on polyphenol and alkaloid contents, on health-promoting constituents, and so on. As cocoa bean fermentations are carried out in the field, in an artisan way on a small scale, or often under nonoptimal conditions on a large scale, the results are variable in terms of quality. In addition, there might be problems of acidity or lack of cocoa flavor (due to poor or incomplete fermentation) and presence of off-flavors (due to overfermented beans and spoilage or poor mechanical drying), all of which lead to low crop value for the farmer. Therefore, it would be desirable to change the fermentation from a whole natural and unpredictable process to a controlled process, initiated with an appropriate starter culture, in which fermentation occurs more quickly and steered. However, one of the reasons that chocolate quality has not been a priority for farmers is that there is no financial incentive to produce high-quality fermented cocoa beans. In the last decades, multinational companies have put most effort into two areas, namely encouraging farmers to maximize production and to improve processing of the fermented cocoa beans. The fermentation process itself has been largely neglected.

The dairy, meat, and alcoholic beverage industries have largely replaced traditional natural fermentations with defined inocula of high-quality raw materials, strict control of the fermentation processes, better treatment of the final products, and diversification of the market (Wood 1998; Leroy and De Vuyst 2004). Cocoa bean fermentations have a long way to go before they reach that stage of development, but preliminary experiments using defined starter cultures in such a complex fermentation process are promising (see below).

### *17.10.2. Experimental Use of Cocoa Bean Starter Cultures*

Schwan (1998) used a defined microbial cocktail inoculum, consisting of one yeast species, *S. chevalieri*; two LAB species, *Lactobacillus delbrueckii*

subsp. *lactis* (formerly *Lactobacillus lactis*) and *Lact. plantarum*; and two AAB species, *A. aceti* and *G. oxydans*, to perform fermentations in 200-kg wooden boxes with aseptically prepared cocoa beans with different inoculation times. The fermentation process mimicked exactly the conditions in 800-kg boxes on Brazilian farms. With the zero-time inoculum, the fermentation was almost identical to the natural one. The fermentation with a phased-addition inoculum was similar, but slower and less pronounced, which led to a slightly poorer end-product. Although preliminary, these data show that the many common species of microorganisms found in natural cocoa bean fermentations can be replaced by a judicious selection and concentration of members of each physiological group that is important for the process. There is, of course, need for improvement in the choice of species and the method of inoculation and fermentation when this technology is to be used commercially. For instance, independent studies on the pectinolytic enzyme, endopolygalacturonase, which is produced by *K. marxianus*, a yeast species isolated from spontaneous cocoa bean fermentations in Bahia, suggest that this yeast species would be a better source of pectinase (Schwan et al. 1995, 1997). Therefore, Schwan (1998) proposed that a combination of *K. marxianus*, as pectinase producer, and the naturally vigorous yeast *S. cerevisiae* as ethanol producer, might be a better yeast choice for future defined inocula. Recently, inoculation of pulp-bean mass with a *K. marxianus* hybrid yeast strain with increased pectinolytic activity has shown that it affects the microbial population structure during fermentation; increases by one-third the volume of sweating, hence naturally increasing aeration; stimulates faster and improved seed protein degradation; and results in reduced acidity, hence positively influencing the fermented cocoa bean quality as well as the sensory quality of the chocolate produced from cocoa beans (Leal et al. 2008).

There have been previous attempts to use yeast and bacterial starter cultures for cocoa bean fermentation, in particular yeasts for an enhanced production of cocoa pulp juice, thereby not affecting the cocoa bean quality (Sanchez et al. 1985; Samah

et al. 1992; Buamah et al. 1997; Dzogbefia et al. 1999). However, with respect to these earlier cocoa bean fermentation studies on starter cultures, no attempt was made to exclude the influence of the natural microbiota. Moreover, pectinolytic (*S. chevalieri*) and non-fermentative (*Candida zeylanoides*) natural yeast isolates or yeasts from culture collections (*Kluyveromyces fragilis*) were tried and no bacteria were added or studied. Furthermore, there was no evidence that the fermentation could be accelerated or improved with these approaches for chocolate production. In the study of Samah et al. (1993), it was shown that the AAB species *Gluconacetobacter xylinus* subsp. *xylinus* (formerly *Acetobacter xylinum*), used as the sole inoculum, produces cocoa beans with higher pH and higher levels of acetic acid; however, a lower chocolate flavor is obtained, as compared with the control beans. Consequently, by setting the initial conditions, defined inocula can now be considered one of the ways that should lead to chocolate of more reliable and better quality.

One of the ultimate purposes of controlled cocoa bean fermentations will be not only to speed up the fermentation process but also to influence flavor and health properties of chocolate products through fermentation. Faster fermentations will be necessary to allow controlled, large-scale fermentation to increase the production capacity to respond to the growing demand for cocoa, although the cocoa crop is in danger worldwide. "Flavor"-controlled fermentations will be necessary to produce marketable, tailor-made cocoa. "Steered" fermentations to maximize the (indigenous) level of health-promoting constituents will allow development of functional chocolate products. In this context, Camu (2007) has shown that the choice of the starter culture does influence the flavor of chocolate made from the concomitant fermented beans, as the fermentation course (speed and population dynamics) and metabolites produced are influenced. This author successfully used starter culture mixes composed of a strain of *Lact. plantarum* (homolactic, citrate-fermenting, mannitol-producing, acid-tolerant), *Lact. fermentum* (heterolactic, citrate-fermenting, mannitol-producing, acid-tolerant), and/or *A. pasteurianus* (lactate-

and ethanol-oxidizing, acetate-overoxidizing, acid-tolerant, and moderately heat-tolerant) to initiate heap fermentations in a natural and artificial environment in Ghana.

### 17.11. Conclusions

To conclude, it will be a challenge to introduce the application of starter cultures in cocoa fermentation practices, either to speed up and better control the fermentation process or to target the fermentation process toward predefined end-products. However, to scale up the initiation of cocoa bean fermentation with a defined inoculum to the farm level, whether applied to small farm holdings or large estates, will be an even greater challenge, especially as it relates to starter culture production and maintenance, inoculation and fermentation methods, and, last but not least, extra costs.

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## Chapter 18

# Microbial Interactions in Kefir: A Natural Probiotic Drink

Graciela L. Garrote, Analía G. Abraham, and Graciela L. De Antoni

*Kefir is a fermented milk originated in the Caucasian mountains obtained by incubation of milk with kefir grains. These grains contain a relatively stable and specific microbiota immobilized in a matrix of polysaccharides and proteins. Numerous species of lactic acid bacteria (LAB), acetic acid bacteria, and yeasts, held together into the matrix, exist in a symbiotic relationship. Microbial interactions in kefir are very complex due to the composition of kefir grains. In this microbial ecosystem, a delicately balanced population of microorganisms occurs, each interacting with and influencing the other members of the population. In kefir grains, the balanced population of microorganisms determines the synthesis of biologically active metabolites that are essential for the grain growth and the inhibition of external microorganisms like pathogens and food contaminants. Although microbial interactions in kefir grains have not yet been very well characterized, it has been established that these interactions are species- and strain-specific. S-layer proteins of Lactobacillus kefir play an important role in co-aggregation between this LAB and Saccharomyces lipolytica as well as in the inhibition of Salmonella adhesion and invasion to Caco-2/TC7 cells. A better knowledge of microbial interaction will be the basis for understanding the kefir grain ecosystem and its probiotic properties.*

### 18.1. General Description of Kefir

Kefir is a fermented beverage originated in the Caucasian mountains, which has become popular in

many European countries. Kefir is a sour fermented milk, sometimes carbonated, with a low alcohol content. It differs from other milk products because it is not the result of the metabolic activity of a single species but of mixed microbiota confined to a matrix of discrete “kefir grains.” They are white, irregular, gelatinous, cauliflower-like structures of variable size ranging from 0.3 to 3.5 cm in diameter. They are composed of proteins and polysaccharides in which the complex microbiota is enclosed. During fermentation, grains increase in size and number, and this is how new biomass is obtained; grains are generally recovered from the fermented milk to be reused.

Despite the wide consumption of kefir, this biological system has not been fully studied, probably due to its complexity. Yeasts, acetic acid bacteria, and lactic acid bacteria (LAB) coexist in a symbiotic association and are responsible for acid-alcoholic fermentation. The activity of the grain depends on the viability of the microbiota. Generally, about  $10^8$  cfu/g of LAB,  $10^6$ – $10^7$  cfu/g of yeasts, and  $10^5$  cfu/g of acetic acid bacteria are present in the kefir grain (Garrote et al. 2001; Witthuhn et al. 2005b). Among LAB, homofermentative and heterofermentative *Lactobacillus*, *Lactococcus*, and *Leuconostoc* are the genera most frequently found. Lactococci, lactobacilli, and yeasts could be mutually stimulated to produce the components of the grain matrix. The distribution of microorganisms within the kefir grain was studied by Bottazzi and Bianchi (1980) using scanning electron microscopy. These authors suggested that the population of

yeasts and lactobacilli were not randomly distributed in the grain. Lactobacilli were located at the periphery of the grain while yeasts were located inside.

The microbiological composition of kefir grains is still controversial. The most common microorganisms isolated from kefir grains are detailed in Table 18.1. Different reports indicate that kefir grain microbiota strongly depends on the grain origin (Ottogalli et al. 1973; Kuo and Lin 1999), on the culture conditions (Molska et al. 1983), and on the storage and elaboration processes (Zourari and Anifantakis 1988; Garrote et al. 1998).

## 18.2. Kefir Grain Preservation

For propagation of the kefir starter culture, a desirable and adequate proportion of the microorganisms composing the kefir grain is required. A decrease in the yeast content of kefir grains alters the rate of biomass production (Garrote et al. 1997). Kefir grains may be preserved lyophilized, dried, or wet. Some authors recommend storage of wet kefir grains at 4°C or drying at room temperature for 36–48 h. Dried kefir grains retain activity for 12–18 months, whereas wet grains retain activity for only 8–10 days (Kosikowski 1982; Marth and Yousef 1991).

**Table 18.1.** Microorganisms found in kefir and kefir grains.

Species	Reference
<i>Lactobacillus kefir</i>	Kandler and Kunath (1983); Marshall et al. (1984); Angulo et al. (1993); Pintado et al. (1996); Takizawa et al. (1998); Garrote et al. (2001)
<i>Lactobacillus kefiranoferiens</i>	Fujisawa et al. (1988); Toba et al. (1991); Mukai et al. (1992); Takizawa et al. (1998)
<i>Lactobacillus kefirgranum</i>	Takizawa et al. (1994, 1998)
<i>Lactobacillus parakefir</i>	Takizawa et al. (1994); Garrote et al. (2001)
<i>Lactobacillus plantarum</i>	Serot et al. (1990); Garrote et al. (2001); Hertzler and Clancy (2003)
<i>Lactobacillus brevis</i>	Ottogalli et al. (1973); Rosi and Rossi (1978); Marshall et al. (1984); Angulo et al. (1993)
<i>Lactobacillus acidophilus</i>	Ottogalli et al. (1973); Angulo et al. (1993); Marshall (1993)
<i>Lactobacillus viridescens</i>	Molska et al. (1983); Angulo et al. (1993)
<i>Lactobacillus gasseri</i>	
<i>Lactobacillus fermentum</i>	
<i>Lactobacillus casei</i>	
<i>Lactobacillus helveticus</i>	Kuo and Lin (1999)
<i>Lactococcus lactis</i> subsp. <i>lactis</i>	Ottogalli et al. (1973); Angulo et al. (1993); Marshall (1993); Pintado et al. (1996); Garrote et al. (2001)
<i>Leuconostoc mesenteroides</i>	Rosi and Rossi (1978); Angulo et al. (1993); Marshall (1993); Kuo and Lin (1999); Garrote et al. (2001)
<i>Acetobacter aceti</i>	Rosi and Rossi (1978); Angulo et al. (1993)
<i>Candida kefir</i>	Zourari and Anifantakis (1988); Engel et al. (1986); Angulo et al. (1993); Marshall (1993); Wyder (2001)
<i>Kluyveromyces lactis</i>	Engel et al. (1986); Angulo et al. (1993); Wyder (2001)
<i>Kluyveromyces marxianus</i>	Rohm et al. (1992); Kuo and Lin (1999); Wyder (2001); Garrote et al. (2001)
<i>Saccharomyces cerevisiae</i>	Rosi (1978); Rohm et al. (1992); Angulo et al. (1993); Marshall (1993); Wyder (2001); Garrote et al. (2001)
<i>Saccharomyces delbrueckii</i>	Rosi (1978); Engel et al. (1986); Pintado et al. (1996)
<i>Saccharomyces unisporus</i>	Engel et al. (1986); Angulo et al. (1993); Wyder (2001)
<i>Torulaspora delbrueckii</i>	Angulo et al. (1993); Wyder (2001)
<i>Candida friedrichii</i>	
<i>Pichia fermentum</i>	Rohm et al. (1992); Angulo et al. (1993); Kuo and Lin (1999); Wyder (2001)
<i>Torulopsis holmii</i>	Wyder (2001)
<i>Zygosaccharomyces florentinus</i>	
<i>Issatchenkia occidentalis</i>	
<i>Yarrowia lipolytica</i>	

Difficulties have been found in maintaining satisfactory quality in grains to produce a beverage with the appropriate and acceptable viscosity. Garrote et al. (1997), comparing two methods for preservation of kefir grains to be employed as starter, evaluated kefir grain metabolic activity when grown in milk after storage and concluded that freezing was the better method for kefir grain preservation. This storage condition maintains the grain activity required for milk fermentation. Since grains stored at  $-20$  or  $-80^{\circ}\text{C}$  showed a greater increase in grain weight after successive sub-culturing, a storage temperature of  $-20^{\circ}\text{C}$  was suggested for household kefir production (Garrote et al. 1997; Witthuhn et al. 2005a).

### 18.3. Methods to Study Kefir Grain Microflora

Conventional methods usually employed in microbiology for identifying and classifying bacteria are mainly based on the analysis of morphological and biochemical properties and are sometimes insufficient for some bacterial groups. Garrote et al. (2001) evaluated the microbiological and chemical composition of four Argentinean kefir grains. The grains microbiota comprised lactobacilli, lactococci, acetic acid bacteria, and yeasts; however, significant differences regarding species were observed. *Lactococcus lactis* subsp. *lactis*, *Lactobacillus kefir*, *Lactococcus plantarum*, *Acetobacter*, and *Saccharomyces* were present in all types of kefir grains, while *Leuconostoc mesenteroides*, *L. lactis* subsp. *lactis* biovar *diacetylactis*, *Lactococcus parakefir*, and *Kluyveromyces marxianus* were grain-specific. These isolates were characterized by traditional microbiological methods including cellular morphology, gas production, and sugar fermentation patterns and growth at different temperatures. Also, the analysis of whole cell protein by SDS-PAGE allowed heterofermentative lactobacilli isolated from kefir grains to accurately identified.

Delfederico et al. (2005) confirmed by molecular methods the identity of 17 heterofermentative lactobacilli isolates obtained from Argentinean

kefir grains. Results of amplified ribosomal DNA restriction analysis from the closely related reference strains studied established the value of this technique for species differentiation of the *Lactobacillus* genus. The data obtained from the analysis of spacer region confirmed that sequencing of this genome region constitutes a reliable tool for the identification of *Lact. kefir* members. In addition, random amplified polymorphic DNA polymerase chain reaction (PCR) patterns allowed the differentiation of isolates (Delfederico et al. 2005).

However, as traditional and molecular methodologies are tedious, expensive, and time-consuming, the application of other techniques has been considered in the last years. Fourier transformed infrared (FT-IR) spectra of intact bacteria are highly specific patterns that may be unique for individual strains. FT-IR spectroscopy is easy to implement, allows analysis of small quantities of biomass, and requires no specific consumables or reagents (Helm et al. 1991; Naumann 2000; Maquelin et al. 2002). Bosch et al. (2006) developed an approach based on FT-IR spectroscopy in combination with multivariate statistical analysis for rapid differentiation of lactobacilli isolated from kefir grains.

Immunological methods have been used extensively to identify bacteria from a variety of ecosystems. The differential enumeration of *Lact. kefir* or *Lact. parakefir* in kefir in viable counts remains very difficult since the colony morphology of these species is similar to other heterofermentative lactobacilli commonly present in kefir. Serological techniques combined with enzyme-linked immunosorbent assays (ELISA) have been used to quantify and distinguish physiologically closely related strains in mixed cultures (Ricke and Schaefer 1990; Durant et al. 1997; Abraham et al. 2005). Garrote et al. (2005) developed an immunochemical assay employing a specific antiserum against *Lact. kefir* S-layer protein to detect and quantify this microorganism in kefir.

Kefir microorganisms that have been isolated using selective growth media and were biochemically and morphologically characterized cannot produce *de novo* kefir grains, indicating that other bacteria are present in this complex microbial



consortia. Molecular techniques offer new opportunities for determining and analyzing the structure and species composition of microbiological communities. Garbers et al. (2004) showed that denaturing gradient gel electrophoresis (DGGE) fingerprinting can be successfully used to typify the microbial consortium present in kefir grains, as well as to distinguish kefir grains cultured using different methods, or kefir grains that have different origins. The grains can be compared with respect to both eubacterial and yeast species present. Wang et al. (2006) demonstrated that PCR-based DGGE and sequence analysis of 16S rDNA proved to be a valuable culture-independent approach for the rapid and specific identification of the microbial species present in micro-ecosystems and probiotic products. The analysis of the obtained bands allowed finding of sequences similar to *Sphingobacterium* sp., *Lactobacillus* sp., *Enterobacter* sp., and *Acinetobacter* sp.

Chen et al. (2008) showed that bacteria that were not isolated by culture-dependent methods were revealed by DGGE. On the contrary, several LAB strains that were previously identified by culture-dependent methods were not detected by PCR-DGGE. The diversity of LAB strains identified by PCR-DGGE was lower than observed by using initial enrichment stage on nutritive media as the cell counts of certain LAB species were lower than the detection limit of PCR-DGGE. Low sensitivities for the detection of the V2–V3 region in a complex environment ( $10^7$ – $10^8$  cfu/g) by DGGE were reported (Fasoli et al. 2003). This detection limitation is a consequence of high quantities of competitor templates of bacteria present in high concentrations. Moreover, various cell proteins and aged culture may interact with the genomic DNA, thereby affecting primer annealing to the template or the activity of the DNA polymerase (de Barros Lopes et al. 1996; Beh et al. 2006).

#### 18.4. Microbial Interactions

Microbial interactions in mixed cultures occur via multiple mechanisms; they may be direct through physical contact or via signaling molecules

(Sieuwerds et al. 2008). The effects of such interactions may either be positive, neutral, or negative and can be divided into five main classes: amensalism, competition, commensalism, parasitism, and mutualism. Amensalism is an interspecies interaction in which one organism adversely affects another organism without being affected itself. It frequently occurs in food fermentations since the major end-products of primary metabolism such as carboxylic acids and alcohols are effective growth inhibitors of indigenous microbiota and spoilage organisms. In the second class of interactions, competition, microorganisms compete for energy sources and nutrients during fermentation. In commensalism, one organism benefits from the interaction while the other strain is not affected. Parasitism occurs when one species benefits at the expense of another. Finally, during mutualism or symbiosis, both participating microorganisms benefit from the interaction. Many food fermentations rely on bacterial interactions; probably the best example of symbiosis is the yogurt consortium, where *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* stimulate in growth and acid production in mixed cultures with respect to single-strain cultures (Sieuwerds et al. 2008).

Which is the real interaction of microorganisms in kefir grains and what is the interaction of kefir microorganisms with exogenous bacteria? The interrelationships of bacteria and yeasts inside kefir grains may have a significant influence on the activities of each strain. The stimulation of growth and lactic acid production by bacteria was observed in the mixed culture; LAB growth was stimulated by growth factors such as vitamins and amino acids produced by yeasts (Simova et al. 2006). Also, bacterial end-products such as lactic acid can be used by yeasts as an energy source. This interaction can be considered symbiotic.

Pure cultures of kefir bacteria and yeasts either do not grow in milk or have a low biochemical activity. Yeasts in kefir provide an environment for the growth of kefir bacteria, producing metabolites that contribute to the flavor and mouthfeel of kefir (Clementi et al. 1989; Simova et al. 2002; Farnworth 2005; Lopitz-Otsoa et al. 2006). Considering the

complexity of the kefir microbiota, all the interactions previously described may coexist to maintain an adequate balance among microorganisms into the grains.

#### *18.4.1. Microbial Interactions and the Biosynthesis of Grain Components*

Kefir grains are clusters of microorganisms held together by a matrix of protein and polysaccharides (Bottazzi et al. 1994; Abraham and De Antoni 1999). Kefir grain matrix is synthesized by the complex microbial population included in kefir grains, which is considered an example of a symbiotic community (Witthuhn et al. 2005b). The main marker for evaluating the symbiotic relationship is the increase in biomass during fermentation. Kefir grains may grow in milk (Garrote et al. 1998, 2001), deproteinized whey (Rimada and Abraham 2001), or soy milk (Abraham and De Antoni 1999; Liu and Lin 2000). To increase the biomass of kefir grains, the synthesis of proteins and polysaccharides is necessary. In this aspect, kefir grain can be compared to a complex biofilm where bacteria and yeasts communication occur through several steps. First, the bacterium approaches to a surface so closely that motility is slowed down. Then, the bacterium may form a transient association with the surface and/or other microbes previously attached to it. Once this association has become stable, the microcolonies formed by bacteria are involved in a three-dimensional biofilm development. Occasionally, the biofilm-associated bacteria detach from the biofilm matrix (Watnik and Kolter 2000). During milk or whey fermentation with kefir grains, different events may be observed. Grains increase their weight as a consequence of the growth of microorganisms and the biosynthesis of grain components, and each type of microorganism grows freely in the growth media. Thus, kefir microbiota associated to grain could be considered a biofilm.

The knowledge about grain proteins, as well as the role of the microorganisms present in grains on the synthesis of the matrix components, is limited. Bassette and Acosta (1988) reported that proteins come from the growth medium (milk). In contrast,

Abraham and De Antoni (1999) demonstrated that protein is produced by the kefir microbiota by bacteria and yeasts associated to the grains. Whole protein profile of kefir grain grown in milk and in soy milk during a long period presented the same pattern in SDS-PAGE, indicating that grain protein does not depend on the growth media (Abraham and De Antoni 1999). Among grain proteins, two kinds of components were distinguished: those easily extractable by dissolving the grain in water and those soluble only after urea/mercaptoethanol treatment. The tightly associated protein may be the one necessary for grain formation. Grain biomass production depends on the time of fermentation (Rimada and Abraham 2001). During whey fermentation, kefir grain biomass increases up to 96 h, indicating that after a certain time of incubation or under certain environmental conditions, grains dissolve partially, releasing their components to the media. Several authors reported that LAB release cytoplasmic hydrolases after certain incubation periods, which could degrade polysaccharides (Gancel and Novel 1994; Pham et al. 2000). Exopolysaccharides are synthesized by kefir microorganisms and are released into the media, reaching values of 218 and 247 mg/l of kefirin in milk and whey, respectively, as reported by Rimada and Abraham (2001, 2003). It was observed that kefir grains produced similar amounts of polysaccharides in deproteinized whey or in milk (Rimada and Abraham 2003), similar to those obtained with other LAB grown in synthetic media (Cerning 1995; Mozzi et al. 2006).

#### *18.4.2. Microbial Interactions and Production of Biological Active Metabolites in Kefir*

Kefir has a long tradition of offering health benefits, especially in Eastern Europe (Zourari and Anifantakis 1988). Several compounds in kefir may have bioactive properties: microorganisms themselves (dead or alive), metabolites produced by microorganisms during fermentation (polysaccharides, bacteriocins), or breakdown-products from the food matrix (peptides); all these compounds may be responsible for the beneficial effects (Farnworth 2005).

**Polysaccharides.** Kefiran, the polysaccharide present in kefir grains, is a water-soluble branched glucogalactan, containing equal amounts of D-glucose and D-galactose, produced by kefir grains or microorganisms isolated from them (Kooiman 1968; Mukai et al. 1988; Micheli et al. 1999). Kefiran has interesting technological applications such as improvement of viscosity and viscoelastic properties of acid milk gels (Rimada and Abraham 2006) as well as formation of films and gels at low temperatures with interesting viscoelastic properties (Mukai et al. 1991; Piermaria et al. 2008). Kefiran films plasticized with glycerol have good mechanical and water vapor properties (Piermaria et al. 2009). Also, several health-promoting properties of kefiran such as immunomodulation (Vinderola et al. 2006), epithelium protection against toxigenic factors from *Bacillus cereus* (Medrano et al. 2008), or antitumoral activity (Murofushi et al. 1983) have been reported for this exopolysaccharide.

The production of kefiran was ascribed to several lactobacillus species that were isolated from kefir grains such as *Lactobacillus* sp. KPB-167B, *Lactobacillus kefirgranum*, *Lact. parakefir*, *Lact. kefir*, or *Lactobacillus kefiranofaciens* (Toba et al. 1987; Fujisawa et al. 1988; Mukai et al. 1988; Yokoi et al. 1990; Micheli et al. 1999; Taniguchi and Tanaka 2004). Recently, a new strain of *Lact. kefiranofaciens* producing up to 1 g/l of kefiran was isolated from Tibet kefir grains (Wang et al. 2008).

Kefiran production by individual strains in different growth media containing wine, alcohol, or whey (Yokoi et al. 1990), or sago starch (Yeesang et al. 2008) was studied to improve kefiran production. A mathematical model was proposed to determine optimal pH profile for the maximum kefiran production in batch cultures, and it was found that the maximum production was obtained at pH 5.0 during the exponential growth phase (Cheirsilp et al. 2001). Kefiran production by a mixed culture of *Lact. kefiranofaciens* and yeasts was studied on a lactose-containing medium. During co-culture of this LAB strain and *Saccharomyces cerevisiae*, it was shown that lactose was converted into kefiran, lactic acid, and galactose by *Lact. kefiranofaciens* (Cheirsilp et al. 2003a). The consumption of lactic acid by

*S. cerevisiae* prevented the accumulation of this compound and therefore the inhibition of *Lact. kefiranofaciens* by this acid (Tada et al. 2007). This fact directly enhances cell growth and kefiran production rates. This co-culture approach has also been described for improving nisin production by a *L. lactis* strain (Shimizu et al. 1999). In addition, kefiran production in a mixed culture under aerobic conditions was higher than that under anaerobic atmosphere (Cheirsilp et al. 2003b). This may be ascribed to the fact that yeasts can grow and produce more growth factors necessary for LAB under aerobic conditions (Lopitz-Otsoa et al. 2006). In addition, the physical contact with *S. cerevisiae* enhanced capsular kefiran production.

In general, optimization of the culture conditions to stimulate production of useful substances by cooperative actions between two microorganisms becomes difficult. Some culture parameters to be considered are pH, temperature, composition of the aeration gas, starter concentration, incubation time, medium nutrient composition, and inoculum percentage of each microorganism (Taniguchi et al. 2001). The development of a mathematical model for kefiran production in a mixed culture constitutes an important tool for defining culture conditions. The model developed by Cheirsilp et al. (2007) considers the impact of *S. cerevisiae* on cell growth, kefiran formation, and substrate assimilation by *Lact. kefiranofaciens*. The construction of mixed culture models for kefiran fermentation allows predicting of the effects of *S. cerevisiae* and environmental factors on growth and kefiran production in a mixed culture.

The production of useful compounds using a single microorganism under culture conditions established by mimicking the actions of yeast cells on *Lact. kefiranofaciens* in kefir grain is an alternative method for optimizing metabolite production. Addition of yeast extract and ethanol, aeration of gas containing CO<sub>2</sub>, and their combinations promotes kefiran production by *Lact. kefiranofaciens* in a single culture (Taniguchi et al. 2001).

**Bacteriocins.** During homemade manufacture, kefir grains are not treated aseptically; in spite of

this, no contamination with undesirable microorganisms has been reported. The high organic acid concentration and the presence of other antimicrobial substances could explain the absence of pathogens in kefir grains manipulated in regular kitchens for thousands of years. Among the antimicrobial substances involved, bacteriocins may be present. Bacteriocins are ribosomally synthesized antimicrobial peptides or proteins (Jack et al. 1995; Ross et al. 2002; for an extensive revision on bacteriocins, see Chapter 5).

A number of lactococci exhibiting antimicrobial activities were isolated from kefir grains in Ireland. The inhibitory substance produced by one of these strains exhibited a broad spectrum of inhibition, similar to that of nisin (Morgan et al. 2000). Also, *Lactobacillus plantarum* ST8KF isolated from kefir produces a 3.5-kDa bacteriocin (bacST8KF) that is active against *Lactobacillus casei*, *Lact. salivarius*, *Lact. curvatus*, and *Listeria innocua* (Powell et al. 2007).

A *Lactococcus* strain isolated from kefir grains produces a bacteriocin, designated lacticin 3147, which displays the advantage of acidifying milk at sufficient rates to allow commercial manufacture of Cheddar cheese. Lacticin is an effective inhibitor of many Gram (+) food pathogens and spoilage microorganisms; thus, starters made with lacticin-producing strains may provide a useful means of controlling the proliferation of undesirable microorganisms during cheese making (Ryan et al. 1996).

#### 18.4.3. Microbial Interactions and Surface Properties

Adhesion of microorganisms to the matrix and co-aggregation between them could have an important role in the maintenance of the number and species balance in kefir grains over time. The microorganisms attached into the grain probably have advantages over free-living microorganisms with respect to survival in stress conditions such as low pH, low nutrient concentration, and suboptimal temperatures (Garrote et al. 1998; Witthuhn et al. 2005b). Bacterial interactions are mediated by polymeric substances that are present on the outside of the cell wall.

Diverse classes of surface constituents have been implicated in bacterial interactions, such as surface exopolysaccharides, surface proteins, lipopolysaccharides, lipoteichoic acids, lectins, S-layers, and fimbriae. The presence of these surface constituents depend on the bacterial genus and strain and the growth and environmental conditions (Navarre and Schneewind 1999).

In bacterial association, ionic or Coulombic interactions, hydrogen bonding, hydrophobic effects, or microbial surface macromolecules such as (glyco) proteins and polysaccharides could be involved. The heterofermentative lactobacilli *Lact. kefir* and *Lact. parakefir* possess S-layer proteins, a macromolecular paracrystalline array of proteins that completely covers bacterial cell surface (Garrote et al. 2004). Yeast surfaces have three major cell wall components, namely glucans, mannans, and chitin (Chaffin et al. 1998; Millsap et al. 1998). Co-aggregation is a process by which genetically distinct microorganisms become attached one to another via specific molecules. Cumulative evidence suggests that such adhesion influences the development of complex multispecies biofilms (Nikolaev and Plakunov 2007). A strong surface interaction between *Lact. kefir* and *Saccharomyces lipolytica* isolated from kefir grains was described. Inhibition of co-aggregation after heating of bacteria and the decrease in the presence of different sugars indicate that the surface molecules involved are thermolabile, suggesting that proteins act as mediators in the aggregation process mediated by a lectin-like activity (Golowczyc et al. 2009).

#### 18.4.4. Microbial Interactions and Probiotic Properties of Kefir

Several health-promoting properties are associated with kefir consumption; in this regard, kefir can be considered a probiotic product. It has been used empirically for the treatment of gastrointestinal and metabolic disorders, atherosclerosis, allergy, and tuberculosis (Saloff-Coste 1996; Lopitz-Otsoa et al. 2006). Several studies demonstrated antitumor activity of kefir (Hosono et al. 1990), stimulation of the immune system (Saloff-Coste 1996; Farnworth

2005), and both antibacterial (Zacconi et al. 1995; Ryan et al. 1996; Garrote et al. 2000) and antifungal activity (Saloff-Coste 1996).

The beneficial action of kefir can be partially attributed to the inhibition of pathogenic microorganisms by metabolic products such as organic acids produced by the kefir microbiota (Garrote et al. 2000). Recent studies demonstrated stimulation of the immune system by kefir (Thoreux and Schmucker 2001; Vinderola et al. 2005) and the role of surface molecules in the protection against enteropathogens (Golowczyc et al. 2008).

The term competitive exclusion was used for the first time by Greenberg (1969) to describe the exclusion of *Salmonella* Typhimurium by normal gut microbiota. Colonization resistance is an analogous term that was introduced by van der Waaij et al. (1971) in studies of the intestinal populations in mice. Microbial interactions and the mechanisms by which indigenous intestinal microorganisms inhibit colonization by invading pathogens are not fully understood. However, *in vitro* and *in vivo* studies suggest that one or more bacterial species may inhibit proliferation or reduce the number of other bacterial types by the following mechanisms (Rolfe 1991): (1) creation of a restrictive physiological environment, (2) production of antibiotic-like substances, (3) competition for bacterial receptor sites, and (4) depletion of or competition for essential substrates. In the development of restrictive environments, the production of organic acids by probiotic microorganisms plays a central role. It is known that lactic acid and volatile short-chain-fatty acids, including acetic, propionic, and butyric acids, inhibit enteropathogens in their non-dissociated state (Helander et al. 1997; Presser et al. 1997).

Most of the studies about the antimicrobial activity of kefir were conducted in *in vitro* experiments. Garrote et al. (2001) demonstrated that kefir obtained with different grains reached a pH of between 3.5 and 4.0 and inhibited the growth of *Escherichia coli*. Thus, milk fermented with grain CIDCA AGK2 was able to halt bacterial growth for at least 25 h. The acid concentration varied between 1.30 and –2.30 g/100 ml in the case of lactic acid, and between 0.13 and 0.29 g/100 ml in the case of acetic acid.

Supernatants of kefir abolished *E. coli* growth in broth. However, yogurt supernatants produced an extension of its lag period. Mixtures of lactic and acetic acids at the concentrations present in kefir also increased the lag time. This study suggests that the inhibitory power of kefir can be attributed to the non-dissociated lactic and acetic acids and other compounds not yet identified (Garrote et al. 2000). On the other hand, Gulmez and Guven (2003) compared the microbiological safety of yogurt and kefir in different combinations by using three food-borne pathogenic strains, *E. coli* O157:H7, *Listeria monocytogenes*, and *Yersinia enterocolitica*, as indicators. They concluded that a combination of yogurt and kefir starter may improve the microbiological safety of the end-product.

The antimicrobial activity of sugar broth fermented with kefir grains against *Candida albicans*, *E. coli*, *Staphylococcus aureus*, *Salmonella typhi*, and *Shigella sonnei* was described by Silva et al. (2009). Ulusoy et al. (2007) qualitatively studied the *in vitro* antimicrobial activity of kefir against *Staph. aureus*, *B. cereus*, *Salmonella enteritidis*, *L. monocytogenes*, and *E. coli*, this activity being stable during storage. The effects of kefir were tested against a toxigenic strain of *B. cereus*. The incubation of milk artificially contaminated with *B. cereus* spores plus 5% kefir grains prevented spore germination and growth of vegetative forms. In addition, the presence of metabolically active kefir grains diminished titers of nonhemolytic enterotoxin A, as assessed by ELISA (Kakisu et al. 2007).

Kourkoutas et al. (2006) evaluated a freeze-dried kefir co-culture as starter for Feta-type cheese production, and they could not detect *Staphylococcus* in the cheese. *Staphylococcus* count was significantly lower in unsalted kefir cheese, not only compared with rennet cheese but more important compared with similar cheeses that had been salt-treated. The supernatants of 11 isolates of *Lact. plantarum* from kefir grains produced strong growth inhibition of *Salmonella enterica* serovar Typhimurium and *E. coli*. However, *Salmonella gallinarum*, *Salmonella enterica* and *Sh. sonnei* were inhibited by some of the strains tested (Golowczyc et al. 2007). Although some strains of



lactococci isolated from kefir produce bacteriocins as mentioned in this chapter, more research is needed to understand the high inhibitory power of kefir supernatants.

Among the criteria suggested for selection of probiotics, the ability to adhere to the gastrointestinal mucosa and colonization has been proposed (Ouweland et al. 1999). This property is strain-specific and is related to the structure and molecular composition of the probiotic cell wall. These characteristics also determine the ability to interact with intestinal mucus, other microorganisms of the enteric microbiota, pathogens orally ingested, and their toxins. Adhesion to mucosal surfaces by probiotics probably protect against pathogens through competition for binding sites and nutrients (Ouweland et al. 2002; Collado et al. 2005) or immune modulation (Salminen et al. 1998). In spite of the lack of definitive proofs, some studies have indicated a relationship between *in vitro* adhesion and *in vivo* colonization (Collado et al. 2007).

Enterohemorrhagic *E. coli* (EHEC) is a food-borne pathogen that causes hemorrhagic colitis and the hemolytic uremic syndrome. Colonization of the human intestinal mucosa that leads to attachment-effacement lesions and Shiga toxin production are critical virulence traits of EHEC. It seems that adhesive type IV pili (EHP) are adherence factors (Rendón et al. 2007) that participate in intestinal colonization (Xicohtencati-Cortes et al. 2007). The effect of probiotics against adhesion of EHEC to intestinal epithelial monolayer was studied *in vitro* with some lactobacilli strains isolated from kefir. Hugo et al. (2008) studied the effect of kefir lactobacilli on the biological activity of EHEC and found that strain *Lact. plantarum* CIDCA 83114, viable or dead, prevented detachment of Hep-2 cells. However, other lactobacilli failed to protect eukaryotic cells. Then, the protective effect was not ascribed to pathogen exclusion and lactobacilli could antagonize virulence mechanisms of EHEC either by modification of the micro-environment or by interfering with the signaling cascades triggered by the pathogen (Hugo et al. 2008).

*Campylobacter jejuni* is recognized as the principal cause of human acute bacterial gastroenteritis. This bacterium occurs at high percentage in poultry, which is the primary source of infection (Harris et al. 1986). Competitive exclusion of this bacterium by kefir was studied in chicks. Zacconi et al. (2003) performed *in vivo* studies to verify the competitive exclusion activity of kefir in chicks by assessing the reduction of *Camp. jejuni* colonization of caecum. They found that fresh and frozen kefir could have interesting applications on the control of the diffusion of pathogenic microorganisms in poultry bleedings.

The ability of bacteria to adhere to mucosal surfaces is important in establishing or maintaining colonization. The bacterial glycocalyx is thought to mediate bacterial adherence to each other and to the intestinal epithelium. Thus, a layer of protective bacteria *could* block the receptor sites for pathogen attachment. The ability to adhere to epithelial cells *in vitro* is a common property of some lactobacilli strains, this property being strain-specific. *Lact. plantarum* and *Lact. kefir* isolated from kefir grains are able to adhere to Caco-2 cells with different percentage of association (0.97%–10% of adhesion). The ability to associate to Caco-2 cells was not related to hydrophobicity since some highly hydrophobic *Lact. kefir* strains (Golowczyc et al. 2007) and some highly hydrophilic strains of *Lact. plantarum* (Golowczyc et al. 2008) were adherent to Caco-2 (0.97%–5.30% of adhesion) in concordance with previous reports (Reid et al. 1994). In contrast, these two properties, adhesion and hydrophobicity, correlate in the genus *Bifidobacterium* (Perez et al. 1998).

In all isolated strains of *Lact. kefir*, the presence of S-layer was demonstrated. S-layer was constituted by a single polypeptide with an apparent molecular mass of 66–71 kDa. This S-layer conferred to the lactobacilli a high hydrophobicity but the presence of S-layer could not be associated with the adhesion to Caco-2 cells. In some strains, the presence of S-layer proteins is associated with the ability to autoaggregate and hemagglutinate. However, in other strains with the same surface structure, S-layer proteins of the same molecular

mass and reactivity against monoclonal antibodies, autoaggregation and hemagglutination were not observed, indicating that other surface molecules could be necessary for the expression of these properties (Garrote et al. 2004; Mobili et al. 2009).

To study the protective action of *Lact. kefir* against adhesion and invasion of Caco-2/TC7, several strains isolated from kefir grains and *Salm. enteritidis* were tested (Golowczyc et al. 2007). In contrast to other reports (Lee et al. 2003), no protection against *Salmonella* was observed with lactobacilli adhered to the cells. However, a significant protection was achieved when lactobacilli and *Salmonella* were previously co-incubated. In this case, *Lact. kefir* CIDCA 8321 co-aggregated with *Salmonella* and had the ability to antagonize the invasion of Caco-2/TC7. In contrast, a non-co-aggregating strain (*Lact. kefir* CIDCA 83113) did not produce any protection. These results suggest that the masking of surface structures of *Salmonella* during co-aggregation interfered with the invasion process. In addition, isolated S-layer proteins from *Lact. kefir* had the ability to autoassemble on the surface of *Salmonella*, preventing the invasion of Caco-2/TC7. It could be interpreted that S-layer proteins interact with specific sites on *Salmonella* surface involved in the first step of mucosal infection or could either modify or mask *Salmonella* structures necessary for the invasion of cultured human enterocytes (Golowczyc et al. 2007).

## 18.5. Conclusions

Microbial interactions in kefir are very complex mainly due to the composition of kefir grains. In this microbial ecosystem a delicately balanced population of microorganisms occurs, each interacting with and influencing the other members of the population. In kefir grains the balanced population of microorganisms determines the synthesis of biologically active metabolites that are essential for grain growth and the inhibition of external microorganisms, like pathogens and food contaminants.

To understand interactions, it is necessary to perform detailed studies on the physiology of the

individual predominating microorganisms to establish their requirements with respect to environmental factors such as nutrients, temperature, pH, oxidation-reduction potential, which may be involved in grain growth, and to determine how these factors affect their preservation and probiotic properties. This information altogether will indicate the possible interactions among microorganisms and will be the basis for understanding kefir grain ecosystem. Extensive research remains to be done on microbial interactions in kefir grains to obtain the desired, precise control of these ecological fermentative processes.

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## Chapter 19

# Safety of Lactic Acid Bacteria

Charles M.A.P. Franz, Gyu-Sung Cho, Wilhelm H. Holzapfel, and Antonio Gálvez

*Lactic acid bacteria (LAB) have a long history of safe use and association with human food production and health. However, in recent years, certain enterococcal strains (mainly of the species *Enterococcus faecium* and *Enterococcus faecalis*) have become recognized as important nosocomial pathogens causing 5%–15% of bacteraemia, endocarditis, and other infections. In contrast, other non-enterococcal LAB (with the exception of the pathogenic streptococci) seldom cause human disease: cases of infection due to lactobacilli and bifidobacteria are estimated to represent about 0.05% to 0.4% of cases of infective endocarditis or bacteremia, while the leuconostocs have been reported to cause even less, that is, <0.01% of bacteraemia cases. Risk factors for enterococcal infection were often noted to occur in patients with underlying heart disease, genitourinary instrumentation, urinary tract infection, abortion, or urinary tract presence of urethral or intravascular catheters, surgery, major burns, multiple trauma, or prior antibiotic therapy. This problem is furthermore confounded by the fact that particular strains exhibit intrinsic or extrinsic antibiotic resistances and that some strains show multiple antibiotic resistance. Some well-known virulence factors and suspected (potential virulence factors) present in enterococci have been located on pathogenic islands. Against the background of the food safety discussion in the European Union (EU), the European Food Safety Authority has launched a “qualified presumption of safety” (QPS) concept, which stipulates that antibiotic safety testing is a requirement for the safety*

*investigation of starter and probiotic cultures in the EU. Research so far has shown that antibiotic resistances in non-enterococcal LAB, which are potentially transferable, occur relatively seldom and that some natural (intrinsic) resistances toward antibiotics occur. Furthermore, problems with antibiotic resistance determinations and evaluations of transfer of resistance determinants clearly require urgent attention, and a great need exists for standardizing media and testing protocols.*

### 19.1. Introduction

The basis for modern food biotechnology was laid during the second half of the 19th century with the first well-founded scientific developments in microbiology. These included, among others, the first description of the lactic acid fermentation by Louis Pasteur in 1857, and the development of the first bacterial pure culture (“*Bacterium lactis*,” Syn.: *Lactococcus lactis*) by Lister in 1873. The early “starter” cultures for fermentations were based on “backslopping” procedures; that is, mixed culture isolates were obtained from earlier successful fermentations. Such former spontaneous fermentations were associated with the (desired) development of the autochthonous microbial population typical of the raw material. These were propagated and handled at the site of production (Mogensen et al. 2002). Such spontaneous fermentations were gradually optimized by different backslopping procedures, which comprise the inoculation of the raw material with a small quantity of the previous successful

fermentation. Backslopping ensures that the dominant strains present in the successful fermentation are used to inoculate fresh raw material and again take over a new fermentation. Backslopping is still used in the production of numerous fermented foods such as sauerkraut, cucumbers, and sourdough (Stiles and Holzapfel 1997; Leroy and De Vuyst 2004), or for products for which the microbial ecology and the role of succession in the microbial population are not well known (Mogensen et al. 2002; Leroy and De Vuyst 2004). Today, in Western countries, however, many fermentations are carried out on an industrial scale (Leroy and De Vuyst 2004), and starter culture production has been industrialized, so that a constant source of pure bacteria is available for reliable fermentations.

The important role of LAB in fermentation is illustrated by their contribution to rapid acidification of the raw material by production mainly of lactic acid. In addition, some strains produce acetic acid, ethanol, aroma compounds, bacteriocins, exopolysaccharides, and important enzymes (e.g., proteases), thereby enhancing the shelf life and microbial safety of the fermented product, in addition to improving the product texture and sensory characteristics (Leroy and De Vuyst 2004).

However, LAB are not only important in food fermentations, but also as probiotics. A beneficial association of LAB with the human host was suggested by Metchnikoff already in 1908). He considered the longevity of Caucasian persons to be related to their high intake of fermented milk products. In contrast to modern perception, Metchnikoff suggested that gut microbes were detrimental rather than beneficial to human health. In addition, he suggested that the substitution of gut microbes with yoghurt bacteria may be beneficial. In this context, LAB and their major metabolite of sugar fermentation, that is, lactic acid, were suggested as health-promoting. Originally defined as microorganisms promoting the growth of other microorganisms (Lilly and Stillwell 1965), probiotics have been defined in recent years as “mono- or mixed cultures of live micro-organisms which, when applied to animal or man, beneficially affect the host by improving the property of the indigenous flora”

(Havenaar et al. 1992), while in relation to food, probiotics are considered “viable preparations in foods or dietary supplements to improve the health of humans and animals” (Salminen et al. 1998). The suggested health-improving properties are still not well understood but are commonly suggested to relate to pathogen interference, exclusion, or antagonism; immunostimulation and immunomodulation; anticarcinogenic and antimutagenic activities; alleviation of symptoms of lactose intolerance; reduction in serum cholesterol; reduction in blood pressure; decreased incidence and duration of diarrhea; prevention of vaginitis; and maintenance of mucosal integrity (Erbringer et al. 1995; Salminen et al. 1996; Klaenhammer and Kullen 1999; Burns and Rowland 2000; Holzapfel and Schillinger 2002; Ouwehand et al. 2002; Isolauri 2004).

LAB are the major representatives of probiotics both on the food and the pharmaceutical markets. As some strains are associated with the human body and occur in the oral cavity, gastrointestinal tract and vagina, this makes these bacteria ideal candidates for application as probiotics. Today, viable probiotic strains supplied in the market mostly contain LAB species in high numbers, either as fermented food commodities or in lyophilized form as supplements or pharmaceutical preparations (Holzapfel and Schillinger 2002). Other microbial species that find application in probiotic products belong to the genus *Bifidobacterium* (*Bifidobacterium adolescentis*, *Bifidobacterium animalis*, *Bifidobacterium bifidum*, *Bifidobacterium infantis*, *Bifidobacterium breve*, *Bifidobacterium longum*), and in addition, strains of *Bacillus cereus* (*toyoi*), *Escherichia coli* (Nissle), and *Saccharomyces cerevisiae* (*boulardii*) have also found application (Holzapfel and Schillinger 2002).

## 19.2. Impact of LAB on the Food Industry

LAB are consumed in enormous quantities, primarily through consumption of fermented foods. According to statistics published in bulletin No. 355 of the International Dairy Federation, the average annual consumption of fermented milk products is

22 kg per capita in Europe. In total, this amounts to about 8.5 billion kg fermented milk per year. With an average microbial content in these fermented products of  $10^8$  bacteria per gram (or milliliter), this amounts to a total of  $8.5 \times 10^{20}$  LAB. Assuming one bacterial cell weighs  $4 \times 10^{-12}$  g, this means that 3400 tonnes of pure LAB cells are consumed every year in Europe. This figure does not take into account the LAB used in other food fermentations such as vegetable and meat fermentations, or especially the probiotic bacteria consumed as supplements or as pharmaceutical preparations. It can thus be expected to be far greater. Today, probiotic foods comprise between 60% and 70% of the total functional food market. Continued increase is observed among the dairy-type probiotic foods, but probiotics are found even in nondairy food products such as fermented meats, and vegetable and fruit juices. Taking into account the wide range of potential (fermentable) substrates, and the different conditions under which LAB strains may be challenged for “functional performance,” it can be expected that developments toward new food-based probiotics will even proceed further in the future (Holzapfel 2005).

### 19.3. Safety and Regulatory Aspects

Internationally, the application of LAB in foods is regulated in different ways and under various categories. These may vary from country to country, and may not necessarily be related to the function of a strain. Under the Food Drug and Cosmetic Act (FD & C Act) of 1958, microbial cultures used in the United States have been defined as “food additives,” following pre-market approval of new uses on the basis of an established standard of safety. However, according to the FD & C Act Section 210 (s), certain classes of substances were explicitly excluded from requirement for Food and Drug Administration (FDA) pre-market approval under Section 409. Among these, substances Generally Recognized as Safe (GRAS) have particularly been mentioned with reference to “their safety under the conditions of its intended use.” A GRAS substance was distinguished from a food additive by (1) widely known informa-

tion relevant to its safe use, and (2) consensus among qualified experts on the established safety of the GRAS substance for its intended use. The FDA thus also lists familiar microorganisms that are considered safe for particular applications. However, it is not the microorganisms that are considered GRAS but rather their traditional use in the food industry.

With two exceptions (Denmark and France), the use of LAB in foods is not regulated by any single harmonized legislation in the European Union (EU), with regard both to their use as starter cultures, as probiotic cultures, or as protective culture and/or food supplements. On the other hand, probiotic cultures for animal feed have been regulated by detailed and fully harmonized EU legislation since 1994. According to Wessels et al. (2004), seven EU laws may determine the use of new LAB strains in foods, comprising Regulation 258/97/EC on novel foods and novel food ingredients, Directive 90/219/EEC on the contained use of genetically modified microorganisms (see Chapter 20); Directive 89/107/EEC on food additives; Directive 88/388/EEC on flavorings for use in foods; Directive 2002/46/EC on food supplements; Directive 95/2/EC on food additives other than colors and sweeteners and Directive 94/40/EC on microorganisms as additives in animal feeding stuffs.

Strangely, there was no recognized means in Europe for microorganisms to be formally considered safe when used for food preparation in the past. In contrast, microorganisms used as feed additives or plant protection products are comprehensively regulated. To explore the possibility of a safety evaluation system similar to the GRAS system, the DG SANCO (EU's Directorate General for Health and Consumer Affairs) Scientific Committees on Food, Animal Nutrition and Plants prepared a working paper for public consultation on a proposed safety approach called “Qualified Presumption of Safety” (QPS), which was open for comment during 2003. This QPS approach represents a possible route to harmonization of approaches for the safety assessment of microorganisms used in food and feed production, without introducing specific measures in areas where there has been no great concern about safety, while allowing more important safety

concerns to be addressed (EFSA 2005a). On the expiry of the mandate of the Scientific Commission for Animal Nutrition (SCAN) early in 2003 and the establishment of the European Food Safety Authority (EFSA) by regulation 178/2002, EFSA took over the task of handling developments of the QPS concept within an EU regulatory framework. The SCAN position paper “Safety Assessment and Regulatory Aspects of Microorganisms in Feed and Food Applications,” which was open for public comment, suggested that a list of specific microorganisms’ history of safe use could be compiled to improve and clarify the present approval of food and feed products produced by such microorganisms. The list would thus be based on a “qualified presumption of safety” (presumption being defined as a belief or assumption based on reasonable evidence and qualified to allow certain restrictions to apply).

Then QPS approach represented as a decision tree where the assessment of microorganisms used as starter cultures mainly requires the following determinations: taxonomy, familiarity, end use, presence of acquired resistance factors, pathogenic potential, and production of undesirable metabolites. Because of the possibility of misunderstandings. The term “familiarity” should be understood as the “body of knowledge” that includes knowledge on history of use, ecology, industrial applications, and scientific literature. Moreover, the safety assessment could be done for a taxonomic group (i.e., genus or group of related species), and if the taxonomic group did not raise safety concerns thereafter, any strain of microorganism for which the identity could be unambiguously established and assigned to the same QPS group would be freed from the need of further safety assessment other than satisfying any qualifications specified (European Food Safety Agency 2007).

In 2007, EFSA asked its Scientific Committee to consider whether this QPS system could be used to harmonize approaches to the safety assessment of microorganisms across the various EFSA scientific panels and if so, the Committee was to develop a strategy for the introduction of an assessment system based on the QPS concept (European Food Safety

Agency 2007). The Scientific Committee reviewed the range and numbers of microorganisms likely to be the subject of an EFSA opinion, and found approximately 100 species of microorganisms that either are or will be referred to EFSA for a safety assessment. A large majority of these species fall into four broad groupings: (1) Gram (+), non-sporulating bacteria (which include the LAB), (2) *Bacillus* spp., (3) yeasts, and (4) filamentous fungi. Accordingly, bacteria, yeasts, and fungi falling within these four groups were selected for an initial assessment of their suitability for QPS status and the Scientific Committee concluded that the weight of evidence for many species falling in the first three groups was sufficient to ensure QPS status. The LAB belonging to group (1) organisms (Gram (+), non-sporulating organisms), which the Scientific Committee considered suitable for QPS status (European Food Safety Agency 2007), are shown in Table 19.1. Where QPS status was proposed, the Scientific Committee was satisfied that the body of knowledge available was sufficient to provide adequate assurance that any potential to produce adverse effects in humans, livestock, or the wider environment is understood and capable of exclusion (European Food Safety Agency 2007). Absence of acquired antibiotic resistance should, however, be demonstrated.

#### 19.4. LAB Associated with Human Infections and Safety Considerations

In view of the large amounts of LAB biomass consumed every year, consideration of the safety of LAB is of great importance. The safety of the microorganisms that have been used traditionally in probiotics has also been confirmed for many strains through a long history of experience. *Lactobacillus* spp., *Lactococcus* spp., *Leuconostoc* spp., *Streptococcus thermophilus* and *Pediococcus* spp. have been used extensively in food processing throughout human history, and consumption of foods containing bacteria and their metabolites has taken place for a long time (Ishibashi and Yamazaki 2001). Until recently, the safety of these microorganisms has not been questioned and reports of



**Table 19.1.** List of lactic acid bacteria proposed for QPS status by the EFSA scientific committee.

Gram-positive, non-sporulating bacteria <sup>a</sup>	Qualifications
<i>Lactobacillus</i> spp.	None
<i>Lact. acidophilus</i> , <i>Lact. amylolyticus</i> , <i>Lact. amylovorus</i> , <i>Lact. alimentarius</i> , <i>Lact. aviaries</i> , <i>Lact. brevis</i> , <i>Lact. buchneri</i> , <i>Lact. casei</i> , <i>Lact. crispatus</i> , <i>Lact. curvatus</i> , <i>Lact. delbrueckii</i> , <i>Lact. farciminis</i> , <i>Lact. fermentum</i> , <i>Lact. gallinarum</i> , <i>Lact. gasseri</i> , <i>Lact. helveticus</i> , <i>Lact. hilgardii</i> , <i>Lact. johnsonii</i> , <i>Lact. kefiranoferiens</i> , <i>Lact. kefir</i> , <i>Lact. mucosae</i> , <i>Lact. panis</i> , <i>Lact. paracasei</i> , <i>Lact. paraplantarum</i> , <i>Lact. pentosus</i> , <i>Lact. plantarum</i> , <i>Lact. pontis</i> , <i>Lact. reuteri</i> , <i>Lact. rhamnosus</i> , <i>Lact. sakei</i> , <i>Lact. salivarius</i> , <i>Lact. sanfranciscensis</i> , <i>Lact. zeae</i>	
<i>Lactococcus</i> spp.	None
<i>L. lactis</i>	
<i>Leuconostoc</i> spp.	None
<i>Leuc. citreum</i> , <i>L. lactis</i> , <i>Leuc. mesenteroides</i>	
<i>Pediococcus</i> spp.	None
<i>P. acidilactici</i>	
<i>P. dextrinicus</i>	
<i>P. pentosaceus</i>	
<i>Streptococcus</i> spp.	None
<i>Strep. thermophilus</i>	

<sup>a</sup> Absence of acquired antibiotic resistance should be systematically demonstrated unless cells are not present in the final product.

harmful effects of these bacteria have been very rare, with the exception of enterococci and pathogenic streptococci. Cases of infection due to lactobacilli and bifidobacteria are rare and estimated to represent about 0.05% to 0.4% of cases of infective endocarditis or bacteremia (Gasser 1994; Saxelin et al. 1996a; Salminen et al. 1998; Borriello et al. 2003). Although such cases therefore are quite rare, they have a high overall mortality rate of approximately 25%, stressing the need for early diagnosis and appropriate treatment (Cannon et al. 2005). *Leuconostocs* have been reported to cause <0.01% of bacteremia cases, but these LAB have also been associated with specific nosocomial outbreaks in Spain (Bou et al. 2008) or India (Taneja et al. 2005). The enterococci are the major exception among the LAB (excluding the pathogenic streptococci), in that these are well known to be important agents of nosocomial disease causing 5%–15% of bacteremia cases (Aguirre and Collins 1993; Gasser 1994; Saxelin et al. 1996a, 1996b; Salminen et al. 1998; Holzapel 2005).

Most LAB that have caused infections in humans belong to the species *Enterococcus faecalis* and *Enterococcus faecium* (Murray 1990; Jett et al. 1994; Johnson 1994; Franz et al. 2003), but other LAB

species such as *Lactobacillus rhamnosus*, *Lactobacillus acidophilus*, *Lactobacillus jensenii*, *Lactobacillus paracasei*, *Lactobacillus casei*, *Lactobacillus curvatus*, *Lactobacillus sakei*, *Lactobacillus salivarius*, *Weissella confusa*, *Leuconostoc mesenteroides*, *Leuconostoc lactis*, *Leuconostoc citreum*, *Leuconostoc pseudomesenteroides*, *Pediococcus Acidilactici*, and *Pediococcus pentosaceus* have also been noted to be associated with human infections (Table 19.2). As reviewed by Cannon et al. (2005), 241 cases of lactobacillemia were identified between 1950 and July 2003, with lactobacilli primarily involved in endocarditis and bacteraemia. *Lact. casei* (35.7%) and *Lact. rhamnosus* (22.9%) were the species most frequently associated with lactobacillemia and infective endocarditis. Generally, most patients from which LAB were isolated had serious underlying disease, which predisposed them to infection. The isolation of LAB from infections is most likely the result of opportunistic infections (Ishibashi and Yamazaki 2001). Risk factors appear to include abnormal heart valves in the case of endocarditis and the presence of a catheter in cases of septicemia (Horwitch et al. 1995; Salminen et al. 1998). Extremes of age and pregnancy appear not to be risk factors for

**Table 19.2.** Overview of reports of disease where lactic acid bacteria were isolated.

Disease	Causative agent	Source of isolation	Reference
Bacteremia	<i>Lact. rhamnosus</i> , <i>Lact. sakei</i> , <i>Lact. curvatus</i> , <i>Lact. salivarius</i> , <i>Lact. fermentum</i> , <i>Lact.</i> <i>paracasei</i> , <i>Lact. plantarum</i> , <i>Leuc. mesenteroides</i> , <i>Leuc.</i> <i>pseudomesenteroides</i> , <i>Leuc.</i> <i>citreum</i> , <i>L. lactis</i> , <i>W. confusa</i> , <i>Lact. jensenii</i> , <i>P. acidilactici</i>	Blood	Sharpe et al. (1973); Mastro et al. (1990); Saxelin et al. (1996a); Antony et al. (1996); Chazan et al. (2008); Svec et al. (2007)
Brain abscess	<i>Leuc. mesenteroides</i>	Abscess	Albanese et al. (2006)
Caries	<i>Lact. paracasei</i> ; <i>Lact. rhamnosus</i>	Sputum	Botha et al. (1998)
Chest infection	<i>Lact. rhamnosus</i>	Sputum	Rahman (1982)
Endocarditis	<i>Lact. rhamnosus</i> ; <i>Lact. curvatus</i> ; <i>Lact. acidophilus</i> , different species of <i>Lactobacillus</i> ; <i>Lact.</i> <i>paracasei</i> , <i>P. acidilactici</i> , <i>Lact.</i> <i>casei</i> , <i>W. confusa</i>	Blood	Fritsche et al. (1973); Sharpe et al. (1973); Tenebaum and Warner (1974); Bayer et al. (1978); Davies et al. (1986); Naude et al. (1988); Griffiths et al. (1992); Harty et al. (1993, 1994); Monterisis et al. (1996); Penot et al. (1998); Vogt and Hoffman (1998); Mackay et al. (1999); Mitchell and Hayek (1999); Barros et al. (2001); Wallet et al. (2002); Flaherty et al. (2003); Ze-Ze et al. (2004)
Endocarditis	<i>Lact. fermentum</i> <i>Lact. acidophilus</i> <i>Leuc. mesenteroides</i>	Mitral valve/ prosthetic valve	Gallemore et al. (1995) Makaryus et al. (2005) Vazquez et al. (1998)
Endocarditis	<i>Lact. rhamnosus</i>	Aortic valve	Golledge (1988)
Empyema of the gallbladder	<i>Lact. rhamnosus</i>	Pus	Allison and Galloway (1988)
Erysipeloid	<i>Lact. rhamnosus</i>	Lymphatic system	Sharpe et al. (1973)
Liver abscess	<i>Lact. acidophilus</i> ; <i>Lact.</i> <i>rhamnosus</i>	Blood and Liver	Klein et al. (1991); Larvol et al. (1996); Rautio et al. (1999)
Pancreatitis	<i>Lact. rhamnosus</i>	Intra-abdominal/blood	Brahimi et al. (2008)
Peritonitis	<i>Lact. rhamnosus</i> , <i>Lact. paracasei</i>	Peritoneum	Klein et al. (1998); Neef et al. (2003)
Pneumonia/lung abscess	<i>Lact. rhamnosus</i>	Sputum	Namnyak et al. (1992)
Pyelonephritis	<i>Lact. jensenii</i>	Urine	Chazan et al. (2008)
Septicemia	<i>P. acidilactici</i> <i>P. pentosaceus</i> <i>Lact. jensenii</i>	Blood	Dickgiesser et al. (1984); Sire et al. (1992); von Witzingerode et al. (2000); Moudden et al. (2007)
Surgical site abscess	<i>Lact. fermentum</i>	Esophagus	Woo et al. (2007)
Urinary tract infection	<i>P. acidilactici</i> <i>Lact. gasseri</i> <i>Leuc. mesenteroides</i>	Urine	Dickgiesser et al. (1984); Barros et al. (2001); Taneja et al. (2005)
Various diseases	Various <i>Lactobacillus</i> species (mainly); <i>Leuc. mesenteroides</i>	Various	Sims (1964); Aguirre and Collins (1993); Husni et al. (1997); Bou et al. (2008)

LAB-associated infection (Adams and Marteau 1995). For enterococci, endocarditis was often noted to occur in patients with underlying heart disease. Risk factors for endocarditis furthermore appear to include preceding genitourinary instrumentation, abortion, and urinary tract infection and instrumentation (Lewis and Zervos 1990; Murray 1990). Risk factors associated with enterococcal bacteremia include underlying disease, presence of urethral or intravascular catheters, surgery, major burns, multiple trauma, or prior antibiotic therapy (Lewis and Zervos 1990; Franz and Holzapfel 2004). For *Pedococcus* infections, risk factors appear to include underlying conditions or underlying malignancy such as diabetes, pulmonary or vascular disease, hyperparathyroidism, burns or trauma, previous antibiotic treatment, abdominal surgery, tube feeding, or, in the last case, also pregnancy (Holzapfel et al. 2006).

Immunocompromised individuals are generally assumed to be more at risk of infection with pathogens and are known to be afflicted with a high incidence of opportunistic infections. However, at least regarding the consumption of probiotic lactobacilli or bifidobacteria, there is no evidence that consumption of probiotic preparations containing these bacteria leads to an increased risk of opportunistic infection among immunocompromised individuals (Borriello et al. 2003). Various clinical studies have been conducted to assess the safety of probiotics in small groups of specific immunocompromised patients (i.e., with HIV infection), and the findings of these studies support the safety of probiotics consumed by such groups (Wolf et al. 1998; Cunningham-Rundles et al. 2000; Borriello et al. 2003; Wessels et al. 2004).

There have been some concerns about the safety of using high numbers of LAB as probiotics in foods. A few rare cases of infection have been associated with LAB used in foods; however, in most cases, a firm connection was not established (Adams 1991). In one case of a liver abscess, the isolate was indeed closely related to a probiotic (Rautio et al. 1999). In another case involving bacteremia, a connection with a combination of chewing a probiotic capsule and dental infection was suggested (Mackay et al. 1999), even though the isolate was not typed

at the molecular level (O'Brien et al. 1999). In another case of *Lactobacillus fermentum*-related endocarditis, the fact of a large daily consumption of milk and dairy products was suggested to play a role, even though no connection with the illness could be proven and no proof of its use in dairy products in general has been shown (Gallemore et al. 1995; Salminen et al. 1998). In a further case, a 54-year-old diabetic woman was reported to develop a *Lact. rhamnosus* septicemia, which was attributed to probiotic use (Zein et al. 2008). Salminen et al. (2002) studied the incidence of *Lactobacillus* bacteremia against the background of a rapid increase in probiotic use in Finland, and could show that this increased probiotic use did not result in a concurrent increase in *Lactobacillus* bacteremia cases. Similarly, Sullivan and Nord (2006) determined the incidence of lactobacilli-induced bacteremia in Stockholm, Sweden, and tried to identify the presence of possible probiotic strains. They found that over a 6-year period the incidence of bacteremia cases remained at the same level, and they could not identify any of the three probiotic strains *Lact. paracasei* F19, *Lact. rhamnosus* GG, or *Lact. acidophilus* NCFB 1748 in any of the clinical samples.

A different situation exists in the case where probiotic bacteria were used in high numbers in a clinical setting in an attempt to cure disease. Thus, *Lactobacillus* sepsis was reported to result from probiotic therapy attempts in children who had severe underlying disease and who developed hospital-associated diarrhea (Land et al. 2005). Thus, attempts to cure the diarrhea led to the invasive disease with the probiotic strains *Lact. rhamnosus* GG (Land et al. 2005). Besselink et al. (2008) attempted to ameliorate the course of predicted severe acute pancreatitis using probiotic therapy. They used a probiotic preparation (Ecologic® 641, Winclove, Bio Industries Amsterdam, The Netherlands) that contained four LAB strains (*Lact. acidophilus*, *Lact. casei*, *Lact. salivarius*, and *L. lactis*) and two *Bifidobacterium* strains (*B. bifidum* and *B. lactis*). Unfortunately, the results clearly showed that this probiotic prophylaxis did not reduce the risk of infectious complications and was even associated with an increased risk of mortality (Besselink et al. 2008).

## 19.5. Antibiotic Resistance of LAB

### 19.5.1. Choice of Media and Determination of Breakpoints

Antimicrobial resistance testing of LAB is a difficult undertaking as testing methods have not been, in the past, standardized to the same extent as, for example, for pathogenic bacteria, because of their relative unimportance as causative agents of human disease. Many LAB require special growth requirements as a result of their adaptation to nutritionally rich environments. Thus, conventional media used for antimicrobial resistance testing such as Mueller–Hinton or Iso-Sensitest media are often not suitable for susceptibility testing of lactobacilli, leuconostocs, or pediococci (Klare et al. 2007). Herra et al. (1995) showed that Wilkens–Chalgren Agar, a medium recommended by the National Committee for Clinical Laboratory Standards (NCCLS) for use when determining antibiotic resistances of anaerobic bacteria, did not support the growth of lactobacilli. However, if Wilkens–Chalgren was supplemented with 5% horse blood, good growth of lactobacilli resulted (Herra et al. 1995). Thus, the choice of media can greatly influence the minimum inhibitory concentration (MIC) values obtained in resistance testing. First, media used for testing antibiotic resistances are chosen to provide an optimal nutritional environment to support the growth of the test microorganism. Second, the medium should provide a suitable gel matrix to allow reproducible and uniform diffusion of the test antibiotic. Third, possible interactions between (un)defined medium components and the antibiotic gradient should be at minimum (Huys et al. 2002). It is well known, for example, that variations in the cation content and the concentrations of critical nutrients such as thymine and folic acid can modify results. Apart from the medium composition, the inoculum size, the incubation temperature, and gaseous atmosphere, as well as the incubation period, may influence antimicrobial resistance testing (Ammor et al. 2007).

In attempts to solve the problem of medium standardization, Klare et al. (2005, 2007) developed a medium called LAB susceptibility test medium, which consists of a mixture of 90% Iso-Sensitest and

10% MRS medium with or without supplementation with l-cysteine for LAB susceptibility determinations. This medium was tested and found to provide the optimum medium in terms of growth support of non-enterococcal LAB and correct indication of MICs of a wide variety of LAB. Overall, the minimum inhibitory concentration values determined in the study of Klare et al. (2005) were in good agreement to those determined with cation adjusted Mueller–Hinton supplemented with lysed horse blood, a medium suggested by the Clinical and Laboratory Standards Institute (CLSI) (formerly the NCCLS) in 2007 for antimicrobial resistance testing of infrequently isolated or fastidious bacteria (Jorgensen and Hindler 2007). Tosi et al. (2007) formulated a *Streptococcus thermophilus* susceptibility test medium (SSM) specifically for testing antimicrobial resistance of *Strep. thermophilus* strains, and this medium allowed a better identification of strains with atypical tetracycline resistances. The SSM medium is a mixed formulation of Iso-Sensitest medium (90%) and M17 medium (10%) supplemented with lactose (0.5% w/v) (Tosi et al. 2007).

Because LAB are such a diverse group of LAB with many genera, each containing many species, it is difficult to specify breakpoints for MIC values that separate the resistant from the susceptible strains. Clearly, some of such breakpoints may be defined at the genus level, when most of the species are known to be resistant or sensitive toward an antibiotic. However, for many LAB genera, this view was found to be too simplistic, as the different species may vary considerably in their susceptibilities toward various antibiotics. The European Commission's Scientific Committee on Animal Nutrition (SCAN 2002) previously supplied a list of breakpoint values for LAB other than enterococci, which was quite preliminary because the breakpoint values given related to the genera *Pediococcus* and *Lactobacillus*, and do not take into account interspecies deviations (Table 19.3). Danielsen and Wind (2003) recognized the shortcoming of this approach when investigating the antibiotic resistances of LAB starter cultures because for some species a natural resistance would require the delineation of a higher breakpoint value when compared to more suscepti-

**Table 19.3.** MIC breakpoint values ( $\mu\text{g/ml}$ ) for different antibiotics according to SCAN (2002), FEEDAP (EFSA 2005b), and Danielsen and Wind (2003).

Antibiotic	SCAN	FEEDAP	Danielsen and Wind
Ampicillin	2	4	4 —
Penicillin G	—	—	4 —
Erythromycin	4	4	L. aci, L. sak, L. curv 1
—	—	—	L. parac, L. rham 2
—	—	—	L. plant, L. pent 4
Chloramphenicol	16	<i>Lactobacillus</i> hom. 4	16
—	—	<i>Lactobacillus</i> het. 4	—
—	—	<i>Leuconostocs</i> , <i>pediococci</i> 4	—
—	—	<i>L. lactis</i> , <i>Strep. thermophilus</i> 8	—
—	—	<i>L. plantarum</i> 8	—
Tetracycline	16	<i>Leuconostocs</i> , <i>pediococci</i> , <i>L. lactis</i> 4	L. para, L. aci, L. rham 4
—	—	<i>Lactobacillus</i> hom. 8	L. sak, L. curv 8
—	—	<i>Lactobacillus</i> het. 8	L. plant, L. pent 64
—	—	<i>L. plantarum</i> 32	—
Ciprofloxacin	<i>Lactobacillus</i> 4	—	— 32
—	<i>Pediococcus</i> 16	—	—
Gentamicin	<i>Lactobacillus</i> 1	<i>Lactobacillus</i> hom. 8	— >128
—	<i>Pediococcus</i> 4	<i>Lactobacillus</i> het. 8	—
—	—	<i>L. plantarum</i> 64	—
—	—	<i>Leuconostocs</i> , <i>pediococci</i> 4	—
—	—	<i>L. lactis</i> , <i>Strep. thermophilus</i> 8	—

L. aci = *Lact. acidophilus*, L. sak = *Lact. sakei*, L. curv = *Lact. curvatus*, L. parac = *Lact. paracasei*, L. rham = *Lact. rhamnosus*, L. plant = *Lact. plantarum*, L. pent = *Lact. pentosus*, *Lactobacillus* hom. = homofermentative lactobacilli, *Lactobacillus* het. = heterofermentative lactobacilli, L. para = *Lact. Paraplantarum*.

ble strains. Thus, while SCAN, for example, gives an MIC breakpoint value of  $1\mu\text{g/ml}$  for gentamicin, Danielsen and Wind (2003) recognized that this was too low and suggested a breakpoint value of  $128\mu\text{g/ml}$  for *Lact. paracasei*, *Lact. plantarum*, *Lact. pentosus*, *Lact. rhamnosus*, *Lact. sakei*, and *Lact. curvatus*, and an even higher breakpoint value of  $256\mu\text{g/ml}$  for *Lact. acidophilus* for this antibiotic (Table 19.3). Similarly, the MIC breakpoint value for streptomycin suggested by SCAN at  $16\mu\text{g/ml}$  was considered too low by Danielsen and Wind (2003), who suggested a value of  $>256\mu\text{g/ml}$  for all LAB. On the other hand, while SCAN recommended a MIC breakpoint value of  $4\mu\text{g/ml}$  for erythromycin, Danielsen and Wind (2003) found this too high to account for observed natural resistances of some *Lactobacillus* spp., and suggested breakpoint values of  $1\mu\text{g/ml}$  for *Lact. acidophilus*, *Lact. sakei*, and *Lact. curvatus*;  $2\mu\text{g/ml}$  for *Lact. paracasei* and

*Lact. rhamnosus*; and  $4\mu\text{g/ml}$  for *Lact. plantarum* and *Lact. pentosus*. In 2005, the FEEDAP panel (Panel on Additives and Products or Substances used in Animal Feed) of EFSA suggested new breakpoint values for 13 different antibiotics and distinguished between breakpoints for obligately homofermentative lactobacilli, heterofermentative lactobacilli, *Lact. plantarum*, *Enterococcus* spp., *Pediococcus* spp., *Leuconostoc* spp., *Lactococcus lactis*, and *Streptococcus thermophilus* (EFSA 2005b) (Table 19.3). In a recent investigation of the resistance of a large number of LAB strains ( $n = 473$ ) belonging to the genera *Lactobacillus*, *Pediococcus*, and *Lactococcus* to 16 antibiotics, Klare et al. (2007) proposed tentative epidemiological cutoff (ECOFF) values for recognizing intrinsic and acquired antimicrobial resistances. Thus, a representative number of strains of at least 12 LAB species were investigated using the microbroth dilution method and



the LSM screening medium. Based on these data, Klare et al. (2007) defined individual ECOFF values for the different antibiotics for each of the 12 species investigated, that is, *Pediococcus acidilactici*, *P. pentosaceus*, *Lact. rhamnosus*, *Lact. paracasei*, *Lact. plantarum*, *Lact. fermentum*, *Lact. gasseri*, *Lact. acidophilus*, *Lactobacillus crispatus*, *Lactobacillus johnsonii*, *Lact. delbrueckii*, and *Lact. reuteri*. Thus, today we have a clearer picture about MIC breakpoint values, which aid in determining whether a strain can be regarded as resistant or susceptible, and will point out highly resistant strains that should be investigated for antimicrobial resistance genes and their transferability, as envisaged by the EFSA QPS safety assessment system.

### 19.5.2. Antibiotic Resistance of LAB

Among the LAB strains that were isolated from human infections, strains of the genus *Enterococcus* are particularly known to contain multiple antibiotic resistances (Klare et al. 1995b). Enterococci are either intrinsically resistant, and resistance genes are located on the chromosome, or they possess acquired resistance determinants that are located on plasmids and transposons (Clewell 1990; Murray 1990; Klare et al. 1995b). Intrinsic antibiotic resistances include resistance to cephalosporins, low levels of  $\beta$ -lactams, sulphonamides, and low levels of clindamycin and aminoglycosides, while examples of acquired resistances include resistance to chloramphenicol, erythromycin, high levels of clindamycin and aminoglycosides, tetracycline, high levels of  $\beta$ -lactams, fluoroquinolones, and glycopeptides such as vancomycin (Murray 1990; Klare et al. 1995b; Leclercq 1997). Vancomycin resistance is of special concern because this antibiotic was considered a last resort for treatment of multiple-resistant enterococci infections. In the mid-1990s, the source of vancomycin-resistant enterococci (VRE) in Europe was shown to be most likely the farm animals as a result of ergotropic use of avoparcin, a glycopeptide antibiotic (Klare et al. 1995a). VRE have indeed been isolated from a wide variety of farm animals, which are important reservoirs of VRE that could be transmit-

ted to the hospital environment via contaminated meat (Swenson et al. 1990; Klare et al. 1995a; McDonald et al. 1997). These findings strongly suggested that food transmission occurred, and as a result the use of avoparcin in animal husbandry was banned in the EU in 1997 (McDonald et al. 1997). In 1999, the streptogramin B/A combination quinupristin-dalfopristin (Synercid®, King Pharmaceuticals, Bristol, TN) was approved in the United States for treatment of VRE. The incidence of streptogramin resistance among *Ent. faecium* isolates from human sources in Europe and in the United States was very low, but streptogramin resistance has been shown to occur frequently in *Ent. faecium* strains of animal (especially poultry) origin (Gallemore et al. 1995). Jensen et al. (2000) and Werner et al. (2002) reported that streptogramin A resistance transferred from farm animals to farmers, suggesting that the frequency of Synercid-resistant *Enterococcus* isolates from humans will rise. Recently, new antibiotics, such as Linezolid, an oxazolidinone, and daptomycin, a novel cyclic lipopeptide antibiotic, have been developed and successfully used for the treatment of VRE. However, the development of resistance continues, and it seems to be merely a matter of time until multiple-resistant *Enterococcus* strains become resistant to these new antibiotics as well.

In our studies on *Enterococcus* isolates from foods (Franz et al. 2001), we showed that resistance toward one or more antibiotics was a common phenomenon among *Ent. faecium* and *Ent. faecalis* strains. *Ent. faecium* strains were mostly (56.3% of strains) resistant to ciprofloxacin, followed by penicillin (45.8%), erythromycin (27.1%), chloramphenicol (10.4%), and tetracycline, streptomycin, gentamicin, and vancomycin at an incidence of <10%. In contrast, *Ent. faecalis* strains were mostly resistant to chloramphenicol (63.8%), followed by streptomycin (46.8%), tetracycline (44.7%), erythromycin (31.9%), ciprofloxacin (27.7%), gentamicin (25.5%), penicillin (12.8%), and ampicillin (2.1%). Teuber et al. (1999) also reported a high incidence of gentamicin-, tetracycline-, chloramphenicol-, and erythromycin-resistant enterococci from foods such as cheeses or fermented meats. Huys et al. (2004) showed that 24% of enterococci

from cheeses were resistant to tetracycline. Giraffa (2002) also reported that enterococci, mainly belonging to the species *Ent. faecalis* and *Ent. faecium*, which were isolated from European cheeses and meat products, showed resistances in different proportions to antibiotics such as penicillin, tetracycline, bacitracin, chloramphenicol, erythromycin, gentamicin, streptomycin, lincomycin, rifampicin, fusidic, acid and vancomycin.

For non-enterococcal LAB, the general picture that is emerging is that antibiotic-resistant LAB can be obtained from both medical and food sources and that some resistances appear to be intrinsic, whereas others appear to be transferable. For vancomycin, the leuconostocs, pediococci, and several *Lactobacillus* spp., especially *Lact. rhamnosus*, *Lact. paracasei*, *Lact. plantarum*, and *Lact. reuteri*, seem to be intrinsically resistant, whereas most *Lactococcus* and lactobacilli belonging to the *Lact. acidophilus* group appear to be sensitive (Danielsen and Wind 2003; Delgado et al. 2005; Ammor et al. 2007; Klare et al. 2007). The sensitivity of *Strep. thermophilus* strains toward vancomycin is variable (Ammor et al. 2007). The resistance of *Lactobacillus*, *Pediococcus*, and *Leuconostoc* spp. to vancomycin is due to the presence of d-ala-d-lactate in their peptidoglycan rather than the d-ala-d-ala dipeptide (Klein et al. 2000).

Most species of *Lactobacillus*, *Lactococcus*, *Streptococcus*, and *Pediococcus* furthermore are resistant to metronidazole as they do not possess hydrogenase activity, which metronidazole targets (Ammor et al. 2007). Furthermore, most *Lactobacillus*, *Pediococcus*, and *Leuconostoc* spp. are resistant to high levels of the cephalosporin cefoxitin. On the other hand, lactobacilli and lactococci are generally sensitive to the inhibitors of cell wall synthesis such as penicillins (piperacillin and ampicillin) and  $\beta$ -lactam antibiotics, but more resistant to oxacillin and cephalosporins (cefoxitin and ceftriaxone, see above). *Strep. thermophilus* strains are highly variable in their susceptibility toward penicillin G and ampicillin (Katla et al. 2001; Temmerman et al. 2003; Ammor et al. 2007). Penicillin G and the  $\beta$ -lactam antibiotic imipenem are usually active against pediococci, while there are

also some reports of resistance of pediococci toward  $\beta$ -lactams (Ammor et al. 2007).

Lactobacilli, leuconostocs, and lactococci are generally susceptible to antibiotics that inhibit protein synthesis such as chloramphenicol, erythromycin, clindamycin, and tetracycline, but more resistant toward the aminoglycosides such as neomycin, kanamycin, streptomycin, and gentamicin (Katla et al. 2001; Danielsen and Wind 2003; Gevers et al. 2003a, 2003b; Temmerman et al. 2003; Delgado et al. 2005; Hummel et al. 2007; Ammor et al. 2007). Specific strains of *Lactobacillus*, *L. lactis*, and *Pediococcus* have been shown to be highly resistant toward chloramphenicol, clindamycin, streptomycin, erythromycin, and tetracycline (Temmerman et al. 2003; Ammor et al. 2007; Klare et al. 2007; Florez et al. 2008). This resistance has been, in many cases, attributed to the presence of resistance genes such as the chloramphenicol acetyltransferase (*cat*) gene in *Lact. plantarum* (Ahn et al. 1992) or in *Lact. reuteri* (Lin et al. 1996). Furthermore, *erm*(B) or *erm*(T) genes encoding resistance toward erythromycin have been characterized in lactobacilli, lactococci, and pediococci (Stroman et al. 2003; Florez et al. 2006; Ammor et al. 2007, 2008). In addition, resistance to macrolides may also be associated with a transition (A to G) mutation in 23S RNA genes (Florez et al. 2007). Tetracycline resistance among LAB is associated with a number of tetracycline resistance genes such as tet(K, M, O, Q, S, W) (Gevers et al. 2003b; Huys et al. 2004; Ammor et al. 2007, 2008; Florez et al. 2008). Recently, Rojo-Bezares et al. (2006) also identified *tet*(L) genes in a *Pediococcus parvulus* strain from wine origin. The aminoglycoside resistance genes *aac*(6')*Ie-aph*(2'')*Ia*, *aaa*(6')*Ie-aph*(2'')*Ia*, *aaa*(6')-*aph*(2''), *aac*(6')-*aph*(2''), *ant*(6), and *aph*(3')-IIIa have been reported to occur in some *Lactobacillus* or *Pediococcus* species (Tenorio et al. 2001; Rojo-Bezares et al. 2006; also see Ammor et al. 2007 for a more complete listing of resistance genes found in LAB). The multiple drug transporters Mdt and LmrP were implicated in the resistance of *L. lactis* to a broad range of antibiotics including lincosamine, streptogramin, tetracycline, and macrolides (Perreten et al. 2001; Putman et al. 2001).

Lactobacilli are usually resistant to most nucleic acid synthesis inhibitors such as enoxacin, perfloxacin, norfloxacin, nalidixic acid, sulphamethoxazole, trimethoprim, and metronidazole (Charteris et al. 1998; Ammor et al. 2007), and such resistances appear to be intrinsic rather than acquired.

Based on these resistance data, it is generally recommended that *Lactobacillus* infections are treated with a  $\beta$ -lactam (penicillin or ampicillin) and an aminoglycoside (typically gentamicin) for synergy, and with erythromycin and/or clindamycin as therapeutic alternatives (Cannon et al. 2005; Danielsen et al. 2007).

## 19.6. LAB "Virulence"

Given the long, that is, thousands of years, safe association of LAB with foods and the very low incidences of association with human disease (excluding *Enterococcus* and *Streptococcus* strains, see above), one really hesitates when using the terms "virulence" or "virulence factors" or "pathogenic" in association with LAB. In the absence of better coined terms that may imply factors that can explain why some LAB can cause disease and allow these bacteria to become associated with an infection, the terms "virulence" and "virulence factors" will be used in the following discussion. Even for the enterococci, virulence factors were long thought to be much more subtle than those of well-recognized Gram (+) pathogens such as the food-associated *Clostridium botulinum*, *Staphylococcus aureus*, *Listeria monocytogenes*, and *Bacillus cereus*. However, considerable progress has been made in the last few years and factors associated with specific stages of enterococcal infection have been well described (Franz and Holzapfel 2004). Johnson (1994) described specific stages for a pathogenic bacterium to cause an infection, that is, the strain should be able to colonize host tissue, resist host-specific and unspecific defense mechanisms, and produce pathological changes either directly, by producing toxin, or indirectly by causing inflammation. Virulence factors of enterococci that are associated with all these four stages have been

studied and identified. These virulence factors are summarized in Table 19.4, and their (possible) roles in the infection causing process are reviewed in Franz et al. (2003) and Franz and Holzapfel (2004). Interestingly, enterococci isolated from foods were shown to harbor either single or multiple virulence factors (Eaton and Gasson 2001; Franz et al. 2001). Eaton and Gasson (2001), however, did show that the incidences of virulence factors among probiotic enterococci strains were noticeably lower than food strains. The fact that enterococci, which can occur from foods, often carry virulence determinants may imply that the association of these bacteria with foods constitutes a safety risk, especially for persons with underlying disease (Eaton and Gasson 2001; Franz and Holzapfel 2004). As mentioned above, it has been noted that VRE transmission in Europe from the community to the hospitals probably occurred via the food chain, thus emphasizing the importance of the food route for transmission of enterococci risk strains that may carry antibiotic resistance or virulence determinants (Franz and Holzapfel 2004).

Because non-enterococcal LAB have a long history of safety with healthy humans and clearly possess an extremely low virulence potential, one is hard stressed to find "virulence factors" among these bacteria. Although it is known that some *W. confusa* strains possess an  $\alpha$ -hemolytic phenotype (Olano et al. 2001), and a dairy strain of *L. lactis* of which the chromosome was sequenced was shown to harbor a gene for a hemolysin III (Bolotin et al. 1999), the impact of these can be described as negligible because such hemolytic characteristics have never been reported to play a major role in the development of an infection. Recently, a number of further LAB genomes were sequenced (Bolotin et al. 1999; Kleerebezem et al. 2003; Pridmore et al. 2004), and information regarding the potential virulence of these bacteria can be searched for within their genomes. The genome of the intestinal probiotic bacterium *Lact. johnsonii* NCC 533 was shown to contain a protein with 50% amino acid sequence similarity to an IgA protease of pathogenic streptococci, which may play a role in hydrolysis of extracellular proteins or in adhesion to the mucosal

**Table 19.4.** Enterococcal virulence factors and their association with stage of virulence or suggested role in disease.

Virulence determinant	(Suggested) association with stage of virulence
Aggregation substance (AS)	Adhesion to eukaryotic cells (adhesin)/promotes colonization Invasion of eukaryotic cells (invasin) Adhesion to extracellular matrix proteins (may promote translocation) Increases survival in immune cells (evasion of host immune response)
Cytolysin (Cyl)	Eukaryotic cell toxin Lyses immune cells (evasion of host immune response)
Gelatinase (Gel)	Can hydrolyze various biological peptides, that is, collagens and fibrin (role in translocation?) Can hydrolyze antibacterial peptides (evasion of host innate immune response)
Enterococcal surface protein (Esp <sub>is</sub> and Esp <sub>im</sub> )	Adhesin, promotes colonization Exhibits characteristics of MSCRAMM's <sup>a</sup> —role in evasion of immune response?
Adhesin to collagen of <i>Ent. faecalis</i> (Ace) or <i>Ent. faecium</i> (Acm)	Adhesion to extracellular matrix proteins (may promote translocation) Exhibits MSCRAMM characteristics: role in evasion of immune response?
Endocarditis antigen from <i>Ent. faecalis</i> or <i>Ent. faecium</i> (EfaA <sub>fs</sub> )	Adhesin: role in endocarditis
Hyaluronidase	Degrades hyaluronic acid, a major extracellular matrix constituent: role in translocation?
Capsule	Evasion of host immune response

<sup>a</sup>MSCRAMM = microbial surface component recognizing adhesive matrix molecules.

surface (Pridmore et al. 2004). IgA proteases prevent bacteria to be trapped in the mucus layer and are thought to be associated with evasion of host immune defense (Pridmore et al. 2004). However, this IgA protease, within the context of this probiotic strain, can also be viewed as a “probiotic property,” especially since other obvious virulence factors are missing. Thus, instead of being associated with virulence, IgA proteases may play an important role in the probiotic bacterium colonizing the gastrointestinal tract.

Vesterlund et al. (2007) studied the presence of putative risk factors in faecal, blood, and probiotic isolates of *Lactobacillus* strains. Thus, the adhesion to extracellular proteins and mucus, hemolysis, ability to avoid the induction of respiratory burst in peripheral blood mononucleocytes, and resistance to human serum were investigated. Among the tested strains, the adhesion to collagen, fibrinogen, and mucus was strain-specific and no significant differences were observed between blood, fecal, and probiotic isolates. The study did not find any clear and

unequivocal virulence factor for lactobacilli; however, the clinical blood isolates showed higher adhesion to mucus than probiotic strains, although the difference was not statistically significant. Furthermore, the authors concluded that further studies should be done on respiratory burst and serum resistance as probiotic strains induced the lowest respiratory burst activity in polymorphonuclear leucocytes and showed the highest resistance against serum (Vesterlund et al. 2007). Also recently, a rat model of infective endocarditis was developed by Vankerckhoven et al. (2007) and the pathogenic potential of clinical isolates of *Lactobacillus* was compared with that of probiotic isolates. In general, their results indicated that probiotic isolates displayed a lower infectivity in experimental endocarditis compared with true endocarditis pathogens. However, the difference in infectivity between *Lact. rhamnosus* endocarditis and probiotic isolates could not be explained by differences in adherence or platelet microbicidal protein susceptibility. Thus, Vankerckhoven et al. (2007) postulated that

other disease-promoting factors may exist in these organisms.

## 19.7. Conclusions

So far, for non-enterococcal LAB, food has not been implicated as a source for isolates that cause human infection. Furthermore, the epidemiological and virulence data suggest that the risk of LAB in fermented foods for causing human infections is negligible, and thus the hazard is very minor for healthy human beings. There are justified concerns about the transfer of antibiotic resistances, however, as LAB from both foods and medical sources have been shown to be potential reservoirs of transferable resistance genes. Thus, the European QPS assessment scheme quite rightly demands the investigation of antimicrobial resistance genes among starter or probiotic strains used in the food supply. Nevertheless, such investigations are still hampered by problems of nonstandardization of methodology when it comes to resistance breakpoint definitions, media for resistance determinations, and experimental protocols for determining transfer rates. Furthermore, recent studies have given reason to discuss whether indeed it makes sense to use probiotics in disease therapy. Given the background of the low incidence of LAB association with human infections, and their historical safety background, one should also bear in mind that the host factors, that is, the health condition and/or immune status of the human being, are determining parameters in the equation of infection with LAB.

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## Chapter 20

# Genetically Modified Lactic Acid Bacteria

Pierre Renault

*Lactic acid bacteria (LAB) are widely used as starter cultures for food production and preservation; their particular fermentative properties improve the safety, flavor, and nutritional quality of the fermented product. In this chapter, diverse strategies (spontaneous mutations, random mutagenesis, or directed modification of the DNA) that are applied to construct genetically modified organisms (GMO) from LAB, as well as their potential use as transforming agents in traditional food processes and in novel applications in the medical or chemical fields, are discussed. Current regulations (in particular U.S. and European legislation) that restrict the use of GMO starter strains with improved traits or with proven health claims, as well as dictate risk assessment procedures and systematic labeling of GMO-containing food products, are compared. Today, new genomic-based technologies allow more accurate prediction and verification of the potential risks associated with genetically modified LAB. The potential benefits that GMO from LAB may offer to the consumers require an update of current legislation, in particular, on the safety assessment procedures for products containing genetically modified LAB.*

### 20.1. Introduction

Lactic acid bacteria (LAB) have been used by man since humans domesticated animals and started to preserve foods. Under the label “lactic acid bacteria,” a quite diverse type of bacteria belonging to the Firmicutes group (low GC% Gram [+] bacteria) is

grouped. The main genera used in the fermented food industry are *Lactococcus*, *Lactobacillus*, *Streptococcus*, *Pediococcus*, and *Oenococcus*. These genera may be quite distant in terms of phylogeny inside the Firmicutes group. Moreover, within each of these genera, often there are species that may be human commensals, pathogens, and environmental bacteria. The genus *Streptococcus* is a good example of this diversity as it includes *Streptococcus thermophilus*, one of the most frequently used starters in dairy technology (yogurt and cheeses); *Streptococcus salivarius*, the most abundant human oral commensal bacteria; and *Streptococcus pneumoniae*, a major human pathogen. Thus, LAB should not be considered a taxonomic designation but as a label of how a group of diverse bacteria is used by humans. Actually, several authors refer to LAB commensal bacteria, such as bifidobacteria, that are Actinomycetales (high GC% Gram [+] bacteria). The production of lactic acid from sugars present in raw materials, and their long history of safe use are among the main properties of LAB (Wood 1997; Chamba and Jamet 2008).

In the last decades, the use of LAB has been rationalized based on the advances in microbiology science. From purely empirical practices (natural contamination and/or backslipping inoculation), the fermented-food industry has developed rigorous processes using well-defined strains selected for particular technological properties. Nowadays, this industry uses selected starter cultures produced and commercialized by international companies. The selection of new starters includes classical

microbiological procedures such as strain purification, species characterization, and technological assays (acidification rates, stress resistance, aroma production, and texturizing properties, etc.).

The industrial importance of LAB has boosted the scientific advance in research areas such as microbiology, physiology, genetics, and more recently, genomics and new high-throughput technologies applications. This knowledge allowed the development of new strategies for improving bacteria use and selection, and in particular, for targeting gene modifications by molecular techniques. Bacteria constructed by using these new techniques are called engineered microorganisms (EM) in the United States and genetically modified organisms (GMO) in Europe. EM in the United States include all types of modifications, such as variants obtained by traditional mutagenesis and laboratory selection.

In this chapter, the diverse strategies to construct GMO and their corresponding denominations are described. The potential use of these organisms, from transformation agents in food processes comparable to traditional uses, to completely new application in the medical field or in fine chemistry, is discussed. GMO are subject to different restrictive regulations depending on the country. In particular, European countries apply extensive risk assessment procedures and systematic labeling of GMO-containing food products. Such regulations are a response to the increasing consumers' concern over these new technologies, whose real usefulness and potential risks are differently perceived. Suspicion about the safety of GMO, as well as political and ethical considerations, restricts the application of such organisms as starter strains constructed by new genetic technologies.

## 20.2. Gene Modification Technologies

Genetic identity, as a factor impacting on the phenotype of all living organisms, is a principle that has been perceived in human society for a long time. This perception may have led humans to select very early the best seeds or animals for improvement of crops and livestock. The results of this selection are

impressive, from aurochs to different cattle varieties with improved meat or milk production, and from triticale to wheat, for example. Very likely, all domesticated races or varieties are the result of profound changes caused by human selection. Thus, the idea of modifying species has been a common practice in farming societies.

### 20.2.1. Spontaneous Modification and Natural Variability

Knowledge and the “scientific basis” of genetic modification of species and races carried out by humans are recent. Mendel's work on peas may be the first milestone in this long quest. Other remarkable milestones are the discovery of DNA; evidence that chromosomes support genetic information (Morgan, Nobel prize in 1933); deciphering DNA structure (Watson and Crick, Nobel prizes in 1962); and decoding genetic information (Nirenberg, Khorana, and Holley, Nobel prizes in 1968). Among significant findings in the microbiology field, the discovery of the “transforming principle” from dead virulent *Streptococcus pneumoniae* strains to living non-virulent strains was reported by Griffith in 1928. Oswald Avery showed in 1944 that DNA was responsible for the transformation event (Avery et al. 1979) allowing *Strep. pneumoniae* to integrate in its chromosome exogenous genetic information. Nowadays, knowledge and know-how have progressed considerably, and scientists are able to introduce new genetic information in most living organisms, including plants, animals, and microorganisms. The organisms produced by these new techniques are commonly called GMO, in contrast to traditional crops and animals, which are considered natural and unmodified. Although the concept of GMO suggests that the ability to modify organisms is new, it should not be ignored that genetic selection in agriculture is millenary. The genome of all living organisms is subject to continuous modifications throughout generations, leading to the diversification, evolution, and adaptation of the organisms to their environment.

The most extensive genetic studies on LAB have been carried out on *Lactococcus lactis*, and, in a

lesser extent, on *Strep. thermophilus* (Davidson et al. 1996; Campo et al. 2002). Recently, the determination of the genome sequence of several strains of the same species of LAB such as *L. lactis*, *Strep. thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus casei*, and *Oenococcus oeni* allowed a direct and global comparison of natural *intraspecies* genetic variability. For example, the two genomes corresponding to the most studied *L. lactis* strains, IL1403 and MG11363, differ significantly (Kok et al. 2005). Only 1775 out of 2300–2500 genes, respectively, are common to the two strains. Thus, over 20%–25% of their genes encode products that likely exhibit different functions. Moreover, shared genes display only 85% average identity, a shift that may lead to significant differences in their functions. Lastly, a comparative genome hybridization study with 47 strains of *Strep. thermophilus* allowed identification of 1271 common genes, showing that the remaining 600–800 genes encoded different functions (Rasmussen et al. 2008).

Bacterial genomes have dynamic structures, shaped by different factors and constraints. They may be extensively rearranged by deletions, duplications, or DNA acquisition by lateral transfer. The main mechanisms leading to these modifications are common to most organisms. Many events appear to be unintended as a result of mutations and rearrangements following errors during DNA replication. In contrast, gene-encoded mechanisms, whose functions appear mostly to generate variability, are also present. All types of modifications are then fixed or lost in the population, depending on the phenotype change that they confer. Genomes therefore tend to lose functions on which no selective pressure is exerted. Modification, including loss of functions, played an essential role in the evolution of many LAB. For example, *Strep. thermophilus* and *Lact. delbrueckii* subsp. *bulgaricus* were subjected to massive losses of genes (Bolotin et al. 2004; van de Guchte et al. 2006). These losses have been estimated to represent over 10% of their initial genome size. A comparable regressive evolution has been observed for microorganisms that colonized new environments, which may be richer in nutrients than their original environments (Andersson and Andersson

1999; Moran and Plague 2004). Thus, *Strep. thermophilus* may have drifted from buccal commensal bacteria and *Lact. delbrueckii* subsp. *bulgaricus* from bacteria living on vegetal materials. In contrast, colonization of a new medium may lead to new constraints generally linked to changes in gene expression levels. For example, spontaneous insertion sequences (IS) have been found in strains of *L. lactis* grown in different conditions (de Visser et al. 2004). Modifications due to IS insertions have also been described in *Lact. delbrueckii* subsp. *bulgaricus* as playing a major role in the adaptation of this bacterium to growth in milk (Lapierre et al. 2002).

Recently, a rapidly evolving and original mechanism of bacterial resistance to bacteriophages was described. In this event, different enzymes that sample small phage fragments and insert them in a dedicated locus called CRISPR, as well as those that use this information to protect the cell against attack of phages displaying identical sequences, are involved. This mechanism generates diversity in a specific locus (Horvath et al. 2008).

Genetic drifts and gene losses cannot really account for the versatility of bacterial adaptation to new niches. Gene acquisition may be among the most important factors that ensure the development of new functions. The concept that such acquisition occurs randomly until the right genes are captured is probably a restrictive view of the reality. Indeed, bacteria have developed strategies to acquire new genes. The recent genomic studies show that gene transfer plays a central role in chromosome shaping. As mentioned before, genome comparison of bacteria belonging to the same species showed that the genetic content of the different isolates could differ over 25%. New genes may be carried out by mobile genetic elements, such as plasmids, transposons, or bacteriophages. For example, in *L. lactis*, many adaptative functions to milk environment are carried out by plasmids (Siezen et al. 2005; Mills et al. 2006; van Hylckama Vlieg et al. 2006). The chromosome is also a target for the insertion of new genes. The genome of *Strep. thermophilus* displays several regions that appear to be preferential insertion docking sites for new genes (Bolotin et al. 2004). For example, this bacterium acquired

methionin and cysteine biosynthetic genes from *Lact. delbrueckii* subsp. *bulgaricus*, which is frequently grown in co-culture with, and phage resistance mechanisms from *L. lactis*. Bioinformatic studies suggested that many gene transfers in several regions of the LAB chromosomes are acquired by lateral transfer, and these observations are further confirmed by the discovery of almost identical genes in genomes of different species.

### 20.2.2. Targeted Modifications

In the last 50 years, two breakthroughs have revolutionized the biology field. In the 1960s, the development of molecular techniques to precisely target changes in genomes by genetic engineering was achieved and, at the beginning of this millennium, genomics allowed a global study of living organisms by integrating their function, diversity, and evolution.

Modification genetic systems may be divided into two processes: (1) transformation, which allows the entry of the new genetic material into the cell, and (2) methods used to select the right events after cell transformation involving the use of selective markers. The first LAB transformations were carried out after protoplastization of *L. lactis* cells to render naked cells, which, when mixed with DNA and a chemical triggering membrane fusion, allowed DNA entry into the cells (Kondo and McKay 1982). Later, electroporation, which uses high electrical fields to modify membrane permeability, was developed and is now widely used for the transformation of many microorganisms (Luchansky et al. 1988). More recently, the discovery of natural competence genes in LAB (Helmark et al. 2004) similar to those of *Strep. pneumoniae* suggests the possibility of developing more efficient transformation systems (Bolotin et al. 2001; Claverys and Martin 2003; Hols et al. 2005). A natural genetic competence system has been manipulated by modifying the expression of a regulator in *Strep. thermophilus* to allow natural competence development in this bacterium (Blomqvist et al. 2006).

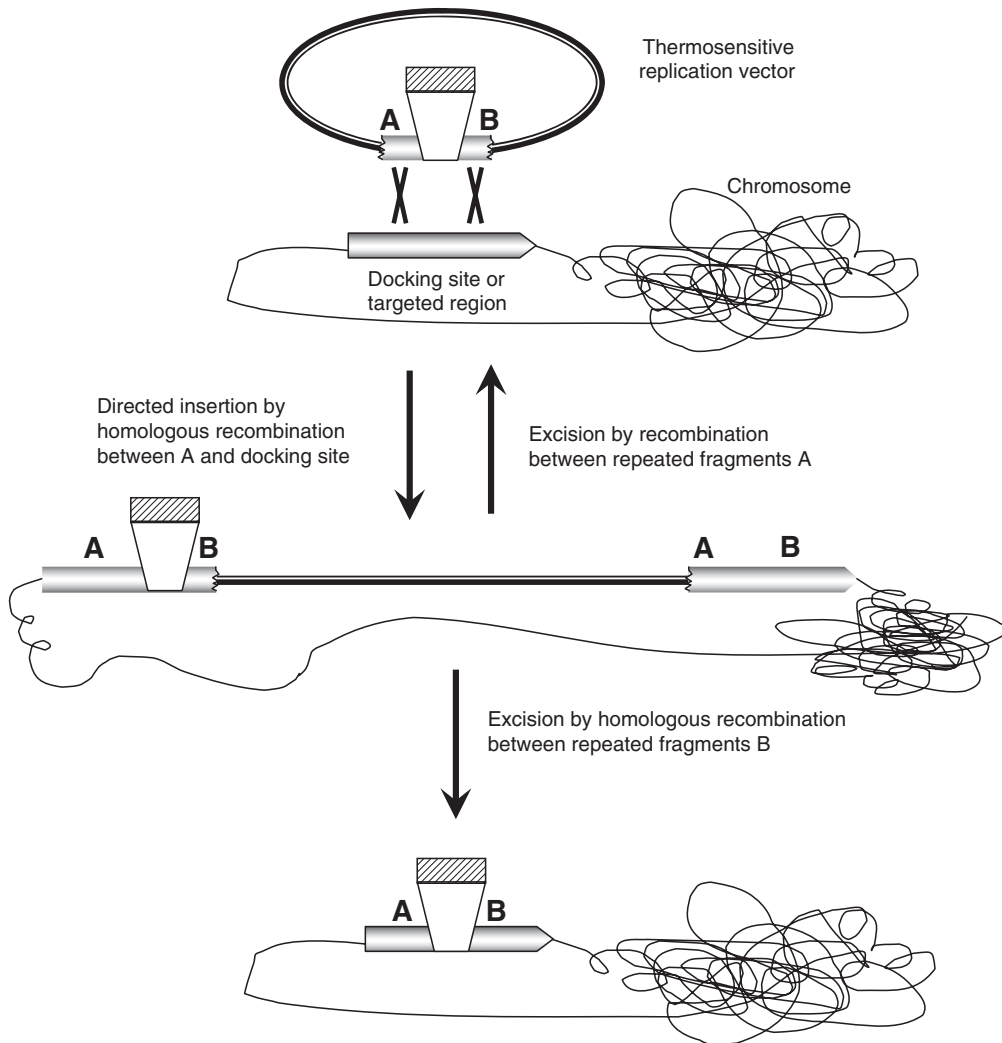
The newly acquired genetic information can be fixed in the genome with the help of different genetic

elements. The use of plasmids and other mobile elements is among the easiest ways to introduce new genes. However, these elements might be unstable and further spread out in nature. Moreover, their introduction and maintenance in the cell require “selective markers” that are supplementary genes to those required for the development of the new targeted functions. Markers used for this purpose could be “food-grade,” meaning they encode genes with functions that are known not to interfere with human health. Antibiotic markers, commonly used in laboratory works, are not considered as food grade (Teuber et al. 1999). Plasmid backbones and markers should come from bacteria of the same species. Different systems have been developed in this way (Henriksen et al. 1999; Renault 1996, 2002). Due to the ingestion of large amounts of bacteria in fermented foods, it is usually preferred to introduce in the modified strains only the necessary genetic information to avoid further lateral transfers. Consequently, genes introduced in modified LAB for food application (in contrast to those developed for research purposes) are usually inserted in the chromosome of their host. One strategy to achieve this task is the use of vectors carrying a conditional replication protein (Biswas et al. 1993). The workflow for this process is illustrated in Figure 20.1. This technique, which allows the introduction of “only-required” genetic information in a precise locus, is indeed an allele replacement. It uses the natural recombination RecA-dependent mechanism, allowing the cell to repair damaged DNA by gene exchange. The modified allele of the docking site may carry a deletion, a punctual mutation, or an insertion of new genes. The engineered modification is usually “structurally” stable and is the exact copy of the modification produced in an easily workable host.

## 20.3. LAB GMO

Two main applications of GMO can be distinguished: food technology, such as quality improvement or technological process reliability, and therapeutic applications, and to a certain extent, probiotics. Although many examples are provided





**Figure 20.1.** Scheme for the construction of targeted mutations in LAB by using thermo-sensitive replication vectors.

(Table 20.1), only a limited number of LAB GMO have been applied beyond laboratory research.

### 20.3.1. Food Applications of LAB GMO

Food fermentation is the traditional application field for LAB. The use of genetic technologies may be foreseen for strain improvement in milk, vegetable, and meat fermentation; however, most examples are provided in the field of dairy technology. Indeed, for

historical and economical reasons, most research has been carried out on dairy LAB, and in particular in *L. lactis*, which became the paradigm for LAB. The knowledge gained on *L. lactis* greatly facilitated the development of LAB application based on GMO technology.

*LAB GMO-based on gene inactivation.* Gene inactivation might be considered as among the simplest cases of genetic engineering leading to the loss

**Table 20.1.** Construction methods of LABGMO and their application fields.

Host	Modified genes	Source of foreign DNA	Modification technique	Phenotype	Application field	Reference
Food bacteria						
<i>Lact. bulgaricus</i>	<i>lacZ</i>	—	Spontaneous mutation by IS	Limitation of lactose fermentation	Less acidic yogurt	Mollet and Delley (1990)
<i>L. lactis</i>	<i>Pip</i>	—	Spontaneous mutation	Inactivation of a membrane protein	Bacteriophage protection	Geller et al. (1993)
<i>L. lactis</i>	<i>aldB</i>	—	Spontaneous mutation	Diacetyl production	Aroma production	Goupil et al. (1996)
<i>L. lactis</i>	<i>ldh</i> and others	—	Chemical mutagenesis	Diacetyl production	Aroma production	Boumerdassi et al. (1997)
<i>L. lactis</i>	<i>aldB</i>	—	Chemical mutagenesis	Diacetyl production	Aroma production	Monnet et al. (1999)
<i>L. lactis</i>	<i>ldh</i> and unknown	—	Chemical mutagenesis	Production of CO <sub>2</sub>	Cheese texture	El Attar et al. (2000)
<i>Lact. delbrueckii</i>	Beta-galactosidase gene	—	Chemical mutagenesis	Lactose utilization	Probiotics, lactose intolerance	Ibrahim and O'Sullivan (2000)
<i>L. lactis</i>	Unknown	—	Spontaneous mutation	Tolerance to acid stress	Robust starters	Rochat et al. (2005a,b)
<i>Lact. lactis</i>	Unknown	—	UV mutagenesis	Increased lactic acid production	Faster acidification	Bai et al. (2004)
<i>Lact. delbrueckii</i>	EPS genes	—	Chemical mutagenesis	Increased EPS production	Texture of fermented products	Welman et al. (2003)
Various LAB	<i>ribC</i>	—	Chemical mutagenesis	Production of riboflavin	Nutrition	Burgess et al. (2004, 2006)
<i>Strep. thermophilus</i>	<i>deoB</i> , <i>gst</i> , <i>rggC</i> , and unknown	—	IS-induced mutation	Tolerance to oxidative stress	Robust starters	Fernandez et al. (2004)
<i>L. lactis</i>	<i>aldB</i>	—	Spontaneous mutation	Diacetyl production	Aroma production	Curic et al. (1999)
<i>L. lactis</i>	<i>aldB</i>	—	Inactivation by bioengineering	Diacetyl production	Aroma production	Swindell et al. (1996)
<i>L. lactis</i>	<i>pip</i>	—	Inactivation by bioengineering	Inactivation of a membrane protein	Protection against bacteriophages	Pedersen et al. (2005)
<i>Lact. helveticus</i>	<i>ldhD</i>	—	Inactivation by bioengineering	Production of highly pure L-(+) lactic acid	Chemical product	Kyla-Nikkila et al. (2000)
<i>Lact. fermentum</i>	<i>ldhD</i> , <i>ldhL</i>	—	Inactivation by bioengineering	Production of mannitol	Additive production	Aarnikunnas et al. (2003)
<i>L. lactis</i>	<i>gdh</i>	<i>L. lactis</i>	Conjugation	Increased alpha-ketoglutarate production	Aroma production	Tanous et al. (2005, 2006)
<i>L. lactis</i> strains	<i>llaI</i> , <i>abiA</i> , <i>abiZ</i> , <i>lac operon</i>	<i>L. lactis</i> strains	Conjugation	Expression of phage resistance mechanisms	Protection against bacteriophages	Sanders et al. (1986)

<i>L. lactis</i> strains	Lactacin genes	<i>L. lactis</i> strains	Conjugation	Production of lactacin	Growth decrease of contaminant	O'Sullivan et al. (2003)
<i>Lact. casei</i> ATCC 334	Dhlc	<i>Lact. casei</i> LC202	Overexpression	Increased alpha-ketoglutarate	Aroma production	Broadbent et al. (2004)
<i>Strep. thermophilus</i>	<i>glyA</i>	<i>Strep. thermophilus</i>	Overexpression	Production of dehydrogenase activity	Aroma production	Chaves et al. (2002)
<i>L. lactis</i>	<i>glk, pfnABCD, pfcBA, genes lactose-PTS and tagatose-6P</i>	<i>L. lactis</i>	Inactivation and overexpression	Modification of acetaldehyde pathway	Aroma production	Pool et al. (2006)
<i>Strep. thermophilus</i>	<i>pgmA, gal U</i>	<i>Strep. thermophilus</i>	Inactivation and overexpression	Modification of metabolic pathway	Texture of fermented products	Levander et al. (2002)
<i>L. lactis</i>	Antisense phase RNA	Lytic phase	Overexpression	Interference with phage gene expression	Protection against bacteriophages	Kim et al. (1992)
<i>L. lactis</i>	Folate gene cluster	<i>L. lactis</i>	Overexpression	Overexpression of folate pathway	Nutrition	Sybesma et al. (2003)
<i>L. lactis</i>	<i>pepN, pepX, pepC, pepI</i>	<i>Lact. helveticus</i>	Overexpression	Modification of proteolytic system	Cheese ripening	Joutsjoki et al. (2002)
<i>L. lactis</i>	<i>pepI, pepL, pepW, pepG</i>	<i>Lact. delbrueckii</i>	Overexpression	Modification of proteolytic system	Cheese ripening	Wegmann et al. (1999)
<i>L. lactis</i>	<i>eps</i> gene cluster	<i>Strep. thermophilus</i> Sft39	Overexpression	Production of EPS	Texture of fermented products	Germond et al. (2001)
<i>L. lactis</i>	<i>eps</i> gene cluster	<i>Strep. thermophilus</i> Sft6	Overexpression	Production of EPS	Texture of fermented products	Stingele et al. (1999)
<i>L. lactis</i>	Phage inducible promoter/LlaI	phi31 lytic phase	Overexpression	Expression of a lethal gene	Protection against bacteriophages	Djordjevic et al. (1997)
<i>Strep. thermophilus</i>	Antisense phase RNA, helicase gene	<i>Strep. thermophilus bacteriophage</i>	Overexpression	Interference with phage gene expression	Protection against bacteriophages	Sturino and Klaenhammer (2002, 2004)
<i>L. lactis</i>	Antisense phase RNA	Lytic phase phi31	Overexpression	Interference with phage gene expression	Protection against bacteriophages	Walker and Klaenhammer (2000)
<i>L. lactis</i>	<i>abiA</i>	<i>Strep. thermophilus</i>	Overexpression	Expression of phage resistance gene	Protection against bacteriophages	Tangney and Fitzgerald (2002)
<i>L. lactis and others</i>	<i>lcnC, lcnD</i>	<i>Ped. acidilactici and others</i>	Overexpression	Production of lantibiotic	Growth decrease of contaminant	Horn et al. (1999)
<i>Lact. plantarum</i>	<i>amyA</i>	<i>Lact. amylovorus</i>	Overexpression	Production of alpha-amylase	Quality of vegetal fermented products	Fitzsimons et al. (1994)

Table 20.1. Continued

Host	Modified genes	Source of foreign DNA	Modification technique	Phenotype	Application field	Reference
<i>Lact. gasseri</i>	folate gene cluster	<i>L. lactis</i>	Overexpression	Increased expression of folate pathways	Nutrition	Wegkamp et al. (2004)
<i>L. lactis</i>	XYL1	<i>P. stipitis</i>	Overexpression	Production of xylitol	Sugar taste	Nyyssölä et al. (2005)
<i>L. lactis</i>	<i>gdh</i>	<i>Peptostreptococcus asaccharolyticus</i>	Overexpression	Increased production of alpha-ketoglutarate	Roma production	Rijnen et al. (2000)
<i>Lact. plantarum</i>	<i>phyC</i>	<i>B. subtilis</i>	Overexpression	Expression of phytase	Quality of vegetal fermented products	Kerovu and Tynkkynen (2000)
<i>L. lactis</i>	<i>lss</i>	<i>S. simulans</i>	Overexpression	Production of lysostaphin	Growth decrease of contaminant	Mierau et al. (2005)
<i>L. lactis</i>	<i>katE</i>	<i>B. subtilis</i>	Overexpression	Tolerance to acid stress	Robust starters	Rochat et al. (2005b)
<i>L. lactis</i>	<i>ldh, alaD, alr</i>	<i>B. spheraicus</i>	Inactivation and overexpression	Modification of metabolism pathways	Alanine production	Hols et al. (1999)
<i>L. lactis</i>	<i>MIPase gene, MtlD, ldh</i>	<i>Eimeria tenella</i> , <i>Lact. plantarum</i>	Inactivation and overexpression	Modification of metabolism pathways	Mannitol production	Wisselink et al. (2005)
<b>Health application</b>						
<i>Strep. mutans</i>	<i>ldh, adh</i>	<i>Zymomonas mobilis</i>	Inactivation and expression	Modification of metabolism pathways	Prevention of tooth decay	Hillman (2002)
<i>Strep. gordonii</i> , <i>Lact. Casei</i>	Protein E7 gene	Human papillomavirus of type 16 (HPV 16)	Overexpression	Antigen production	Vaccine against papillomavirus	Di Fabio et al. (1998); Medaglini et al. (1998)
<i>L. lactis</i>	<i>Csp</i>	<i>S. pneumoniae</i>	Overexpression	Production of capsular polysaccharide	Vaccine against <i>Streptococcus pneumoniae</i>	Gilbert et al. (2000)

<i>L. lactis</i>	L7/L12 protein gene	<i>Brucella abortus</i>	Overexpression	Antigen production	Vaccine against <i>Brucella</i>	Ribeiro et al. (2002)
<i>L. lactis</i>	Rotavirus gene	Rotavirus bovin	Overexpression	Antigene production	Vaccin against rotavirus	Enouf et al. (2001)
<i>Lact. Plantarum</i>	DNA encoding gp41E	Virus HIV	Overexpression	Antigene production	Vaginal immunization for HIV	Hols et al. (1997)
<i>Strep. godonii</i> , <i>Lact. Casei</i>	DNA encoding gp120 V3 domain	Virus HIV	Overexpression	Antigene production	Vaginal immunization for HIV	Di Fabio et al. (1998); Medaglini et al. (1998); Oggioni et al. (1999)
<i>L. lactis</i> , <i>Lact. Plantarum</i>	DNA encoding tetanic toxin fragment C	<i>Clostridium tetani</i>	Overexpression	Production of tetanic toxin fragment C	Protection against tetanic toxin	Wells et al. (1993); Norton et al. (1997); Robinson et al. (1997); Grangette et al. (2001)
<i>Lact. Plantarum</i>	Gene fragment of choleric toxin B	<i>Vibrio cholerae</i>	Overexpression	Antigene production	Protection against choleric toxin	Slos et al. (1998)
<i>L. lactis</i>	IL-10	Mice	Overexpression	Production of cellular mediator	Bowel disease treatment	Steidler et al. (2000); Steidler (2001); Braat et al. (2006)
<i>L. lactis</i>	beta-lactoglobulin gene	Cow	Overexpression	Antigene production	Tolerance to food allergy	Chatel et al. (2001)
<i>L. lactis</i>	<i>lipA</i>	<i>Staphylococcus hyicus</i>	Overexpression	Production of a lipolytic enzyme	Steatorrhea treatment	Drouault et al. (2000, 2002)
<i>Strep. godonii</i>	Gene encoding antibody chain	—	Overexpression	Production of an antibody chain	Treatment of vaginal infection	Beninati et al. (2000); Oggioni et al. (2001)
<i>L. lactis</i>	TFF	Mice	Overexpression	Production of cellular mediator	Treatment of acute bowel disease	Vandenbroucke et al. (2004)



of functions. This type of modification may lead to applications when functions interfere with interesting pathways, or directly perform unwanted processes. Examples on *aldB*, *pip*, and undesirable genes in starter strains are given below.

The *aldB* gene encodes acetolactate decarboxylase (ALDC), which transforms acetolactate into acetoin (see Fig. 20.2 in the color plate section). Acetolactate, produced from two pyruvate molecules, is involved in two different metabolic pathways: first is in the synthesis of valine and leucine, and the second is in the catabolism of pyruvate into acetoin and butanediol. Lastly, acetolactate is a precursor for diacetyl formation, an aroma compound that gives a buttery and creamy flavor to fresh dairy products such as butter, cream, and buttermilk. Since this metabolite is produced in small amounts and is further degraded into acetoin, food products rapidly lose this pleasant aroma. The weak production of diacetyl is mainly attributed to ALDC activity. Indeed, in the presence of ALDC, acetolactate is rapidly converted into acetoin, which is then converted into butanediol, both of which are not aromatic and do not contribute to improvement of product quality. Acetolactate is therefore not available in sufficient amount to allow significant production of diacetyl. Examination of this metabolic pathway suggests that inactivation of ALDC would allow acetolactate to accumulate and therefore be converted into diacetyl. This hypothesis was tested by introducing mutations in *L. lactis* subsp. *diacetylactis* *aldB* gene. Different methods were used for this purpose: (1) chemically induced mutants by using N-methyl-N'-nitro-N-nitrosoguanidine (Monnet et al. 1997; Aymes et al. 1999); (2) spontaneous mutants (Goupil et al. 1996; Curic et al. 1999), and (3) genetically engineered mutants (Swindell et al. 1996). Regardless of the applied techniques, *aldB* gene mutants displayed a similar improved diacetyl production. However, the different mutants were not equivalent with regard to food regulation. For example, spontaneous mutants obtained by using a valine-synthesis rescue plasmid (which is cured after mutant selection) are considered GMO in the EU but substantially equivalent to the natural variant in United States.

In terms of reliability, the different procedures are not similar. The classical mutagenesis procedure, which uses chemical or physical mutagenic agents, is known to produce additional random mutations in the genome. To select a clone, different screenings are thus necessary to obtain bacteria having the proper phenotype. In general, the first screening is directed on the new trait to be isolated, and then other screenings are carried out to check that the mutant still possesses relevant parental properties (e.g., growth acidification). However, it is still possible that the mutant carries supplementary mutations that were not apparent in further screenings. Concerning the valine-dependent selection, cells are transformed by a plasmid which is removed after the mutant selection procedure. The selection itself is based on growth ability on a chemically defined medium, a procedure that is not supposed to produce side mutagenesis. Practically, it is not possible to "certify" that no additional mutations arise. Spontaneous mutations occur at a lower rate (one to 10 errors in every  $10^8$  nucleotides replicated) than chemically induced mutations, although it is not possible to rule out that spontaneous mutagenesis may increase as some SOS systems may be triggered under trophic limitations (Delmas and Matic 2005). An increase in the mutagenesis rate has been described as "adaptive mutagenesis" and may be induced by nutritional stress (Bjedov et al. 2003; Tenaillon et al. 2004). However, the adaptive mutation rate remains much lower than those induced by chemical mutagenesis.

Inactivation of *pip* gene provides a second example of gene inactivation (see Fig. 20.3 in the color plate section). This membrane protein is required for *L. lactis* infection by bacteriophages (Geller et al. 1993). This protein might be involved in phage adsorption to cell before DNA injection. Deletion mutant for Pip is thus phage resistant, a feature that is particularly important for industrial use of starter cultures. Indeed, most bacteria used for fermentation may be infected by phages that kill them, leading to fermentation arrest and loss of product quality. Different strategies may be used to minimize the impact of a phage attack, although none provides long-term reliable protection. Indeed,

no strains have been found to be resistant to all bacteriophages. However, each bacteriophage has a particular host range at strain level; thus, by combining strains displaying complementary resistance properties, the risk of infection is minimized. In this respect, generating strains having a new type of phage resistance may be considered part of a more global strategy to limit phage impact. Isolation of spontaneous or mutagenesis-induced phage-resistant mutants affected in the *pip* gene demands specific testing to discard mutants affected in other relevant genes. On the contrary, targeted mutagenesis allows the construction of a well-defined *pip* mutant (Pedersen et al. 2005).

In addition, gene inactivation can also be used to remove undesirable properties, such as antibiotic resistance and biosynthesis of biogenic amines, from starter cultures. The presence of antibiotic resistance genes is not desirable in starter strains, even when these genes are found naturally. Their impact on health is not direct since their presence does not lead to harmful products. However, it is now generally accepted that their dissemination to other bacteria, and incidentally to pathogens, decreases the efficacy of antibiotic treatments. Therefore, undesirable genes should be absent from genetically modified strains and moreover from starters used in food processes. In this sense, elimination of undesirable genes is required when using new interesting technological strains isolated from foods (Teuber et al. 1999). A second example is the inactivation of genes involved in the biosynthesis of biogenic amines. Accumulation of biogenic amines in food may be a result of microbial metabolism by decarboxylases acting on amino acids such as histidine and tyrosine. These decarboxylases are present in bacteria from the genera *Lactobacillus*, *Enterococcus*, *Carnobacterium*, *Pediococcus*, *Lactococcus*, and *Leuconostoc* (Stratton et al. 1991; Lonvaud-Funel 2001; Suzzi and Gardini 2003). Different techniques are available to detect bacteria having these undesirable genes (Coton and Coton 2005; Marcobal et al. 2005; Costantini et al. 2006). Several undesirable genes are carried by mobile elements, which can be eliminated by simple treatment. For example, resistance cassette embedded between

repeated insertion sequences or in plasmids can be removed by heat treatment (Stroman et al. 2003; Chin et al. 2005; Huys et al. 2006). As has been described for antibiotic resistance genes, it may be possible to isolate cured variants of genes involved in the synthesis of biogenic amines (Lucas et al. 2005). This strategy may be limited by the fact that the undesirable genes may be inserted in the chromosome as shown in *Enterococcus* (Connil et al. 2002). Otherwise, bioengineering would allow removal of these genes for most interesting technological strains.

*LAB GMO-based on expression of new genes.* In the preceding section, we showed the interest of genetic engineering approaches to remove genes carrying undesirable functions. Likewise, these techniques could be used to introduce new functions, which may be initially encoded by genes coming from closely related bacteria, or by phylogenetically more distantly related organisms. This distinction has no real importance in terms of engineering, but may have an important impact in terms of use and commercialization of the new organisms.

Introduction of genes from donors of the same species is the most frequently described technique for strain construction. There might be different reasons to introduce one or more genes isolated from closely related microorganisms in a given strain. First, genes carrying out functions of interest may be present at low frequency in a population. Engineering would allow enlarging the number of strains displaying a particular phenotype in the same species. Second, genes encoding for a given function might be present, but their expression could not be optimal in food processes. When genes of interest are carried out by mobile elements, traditional methods, such as plasmid conjugation, may be used to transfer functions (Sanders et al. 1986). However, this possibility is relatively restricted compared with examples of bioengineering using food grade vectors, or after gene insertions in the chromosome as shown Table 20.1.

Genes of particular interest to be introduced in new strains include phage resistance mechanisms,

metabolic functions (lactose and citrate assimilation, exopolysaccharide synthesis, etc.), bacteriocin production, and so on. Interestingly, these functions are also often carried by mobile elements or have been acquired naturally by horizontal transfer. For example, several restriction/modification mechanism genes (phage resistance) are identical in *L. lactis* and *Strep. thermophilus*. Moreover, several *Strep. thermophilus* *eps* operons contain genes found on *L. lactis* plasmids (F. Rallu et al., pers. comm.). Modified genes are often placed on plasmid vectors and controlled by specific promoters. Nevertheless, they can also be introduced in the chromosome. In several cases, punctual mutations, equivalent to spontaneous mutations, may be introduced to modify the level of expression of natural promoters.

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Moreover, many heterologous genes have been expressed in LAB (Table 20.1). In most cases, these genes come from phylogenetically closely related species. However, distantly related bacteria (often from other low GC% Gram [+] bacteria) as well as yeasts and other eukaryotes (*Pichia stipitis*, *Eimeria tenella*, etc.) have been used as genes sources. The introduction of totally new functions might be based on the expression of a unique enzyme allowing the utilization of new substrates or the expression of a complete set of genes to constitute a complete biosynthetic (e.g., vitamins, exopolysaccharides) pathway.

### 20.3.2. Health Applications of LAB GMO

Certain LAB GMO have been designed for health applications (Table 20.1). For example, phase I clinical trials were performed with a *L. lactis* strain producing interleukine 10 for chronic intestinal disease treatment (Baat et al. 2006). In the field of dental care, trials have also been performed to evaluate strategies against the development of cariogenic strains of *Streptococcus mutans* by competition with harmless derivatives unable to produce lactic acid, the acidifying compound that leads to tooth decay (Hillman 2002). Also, several animal trials have been carried out with other types of LAB GMO, and some encouraging results have been

obtained, for example, in the vaccination field (Chapter 9 of this book) or in the supply of enzymes in human diseases (Drouault et al. 2002).

### 20.3.3. Other Applications

LAB may also produce metabolic compounds that have application as food additives or chemicals. For example, a strain of *L. lactis* has been designed to produce L-alanine instead of L-lactate (Hols et al. 1999). The design of this bacterium allowed the rerouting of pyruvate catabolism, usually yielding lactate, toward alanine production. It was constructed by expressing alanine dehydrogenase, an enzyme absent in *L. lactis*, with the gene from *Bacillus sphaericus*. This bacterium produces both alanine and lactate from pyruvate. To optimize alanine production, the *ldh* gene, encoding the enzyme allowing the formation of lactate from pyruvate, was inactivated to exclusively produce alanine from pyruvate. Finally, since only the L-alanine form is desired, the gene encoding the racemization between the two isomer forms was also inactivated. The resulting bacterium transformed 99% pyruvate into L-alanine. The purified L-alanine can be used as a food additive or in pharmaceutical preparations.

In a completely different area, a kit for the detection of antibiotics in milk is based on the use of a *Strep. thermophilus* strain expressing the luciferase gene (Jacobs et al. 1995); the marketing of this strain is allowed by EC directive 90/220/EEC. In this application, *Strep. thermophilus* is used as a sensor since its growth is strongly affected by the presence of antibiotics in the medium. In antibiotic free media, the metabolism of the modified strain is active and allows the expression of the luciferase gene, and emission of light. On the contrary, in media such as milk contaminated with antibiotics, growth cell is inhibited or retarded and no light is emitted. This test allows the early detection of antibiotics and other inhibiting substances in milk that are forbidden or can interfere with milk processing. Noteworthy, in this application, no GMO is released in nature since the test can be carried out in the laboratory with few milliliters of milk.

## 20.4. Regulation, Risk Assessment, and Acceptability of LAB GMO

Design of LAB GMO allows the improvement of processes or the development of new products. However, like for any technological innovation, risk assessment procedures are required prior to large-scale use of the modified strains, their dissemination in the environment, and ingestion by consumers. The general outline of risk assessment takes into account the type of modifications carried by the GMO, their consequences on the physiology of the bacteria, and, finally, on the use the new microorganisms. Most countries have established specific regulations for risk assessment and use of GMO. Types of modifications; risk assessment tests; and types of information on the labels of commercialized GMO-containing products may significantly differ depending on the country.

### 20.4.1. What Is a GMO: The Regulatory Views

Table 20.2 summarizes the different types of modifications introduced in food microorganisms used in the dairy industry. As stated above, the definition of GMO may significantly differ among countries. In the EU, GMO means an organism “in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination” (Directive 2001/18/EC). Therefore, bacteria modified by mutagenesis, conjugation, transduction, or even natural transformation are not GMO as long as modifications have not been constructed by molecular techniques. Techniques for “genetic engineering” are defined as involving methods for the direct introduction of heritable genetic material prepared outside of the organism. GMO are thus organisms containing modifications, which at least in one step, were performed *in vitro*. This definition is relatively close to that given in New Zealand, where GMO are defined as organisms that were designed with molecular techniques, regardless of the type of modification conducted (introduction of new genes or modification of already existing genes).

In the United States, genetic modification is an expression used in a larger context and includes every organism displaying any genotypical change. Therefore, any nucleotide change resulting from the use of traditional or molecular techniques yields a GMO. This expression is therefore not used to discriminate microorganisms produced by new technologies from the traditional ones. Organisms having new properties and produced by molecular techniques are called “bioengineered.” Regulation focuses therefore on the properties yielded by the modification rather than on the way the organisms were designed. Therefore, there is a consensus to submit microorganisms containing DNA originating from organisms belonging to different species to a specific regulation involving a risk assessment procedure before any marketing. This procedure aims to certify that the expression of the new genes does not yield, for example, new chemical, toxicological, and microbial risk. Moreover, natural bacteria and their variants obtained by classical methods are not submitted to specific risk assessment, provided there is evidence that the strains belong to species that have a long history of safe use. Strains belonging to new species or strains whose applications significantly differ from traditional use are evaluated by the procedure defined in “novel food” regulation. This approach allows, to a certain extent, the application of the principle of “substantial equivalence,” which consists of, in the frame of well-defined knowledge, strains of these species that behave similarly to the relative species already approved as safe. Finally, there are divergences on the assessment of microorganisms built using molecular techniques without containing foreign DNA. Striking case studies are the *pip* and *aldB* *L. lactis* mutants previously described (Fig. 20.4). In the U.S. regulation, these mutants are considered substantially equivalent to natural strains, whatever the technique used to produce them (in this case, the strains do not contain any foreign DNA). Therefore, they can be industrially used without the need of a specific assessment procedure. In the European regulation, mutants produced by bioengineering are regarded as GMO and will be evaluated by a broad risk assessment procedure similar to those expressing new

**Table 20.2.** Sources and main properties of food microorganisms used in the dairy industry.

	Source	Genetic features	Acceptability in food	Labeling
Natural strain	Isolated from food products or from the environment. Their origin before screening is generally unknown	Defined at the species level. The species are usually known to have a long history of safe use for traditional applications. The intraspecies genetic content may vary between 1% and 25%	Accepted without further safety evaluation once the strains belong to recognized food species used in similar products	No requirement for specific labeling, except eventually the name of the species used in the process
Variants obtained by classical techniques	Selected in R&D from already used strains	Genetic content modified by nondirected techniques (chemical mutagenesis, UV) Generally, the mutations are punctual, although larger rearrangements could also occur. It also includes plasmid and mobile elements transferred by conjugation or transduction	Accepted without new assessment since they are considered substantially equivalent to already used strains	No requirement for specific labeling
Variants constructed in laboratories	Designed in R&D laboratories from already used strains with molecular methods (nonfood strains may be used for construction and the presence of recombinant DNA in the resulting strain)	Targeted genetic modifications. Absence of heterologous DNA	Different status in the world: – US: “substantially equivalent” to natural strains – EU: case by case safety evaluation, like GMO	Different status in the world: – US: no specific labeling – EU: labeled GMO
Transgenic strains	Constructed by molecular techniques; containing foreign and recombinant DNA	Targeted genetic modifications. Presence of heterologous DNA	Case by case safety evaluation in the frame of GMO legislation in the EU or bioengineered strains in the United States	Different status in the world: – US: bioengineered strain with specific characteristic compared to reference strains – EU: labeled GMO

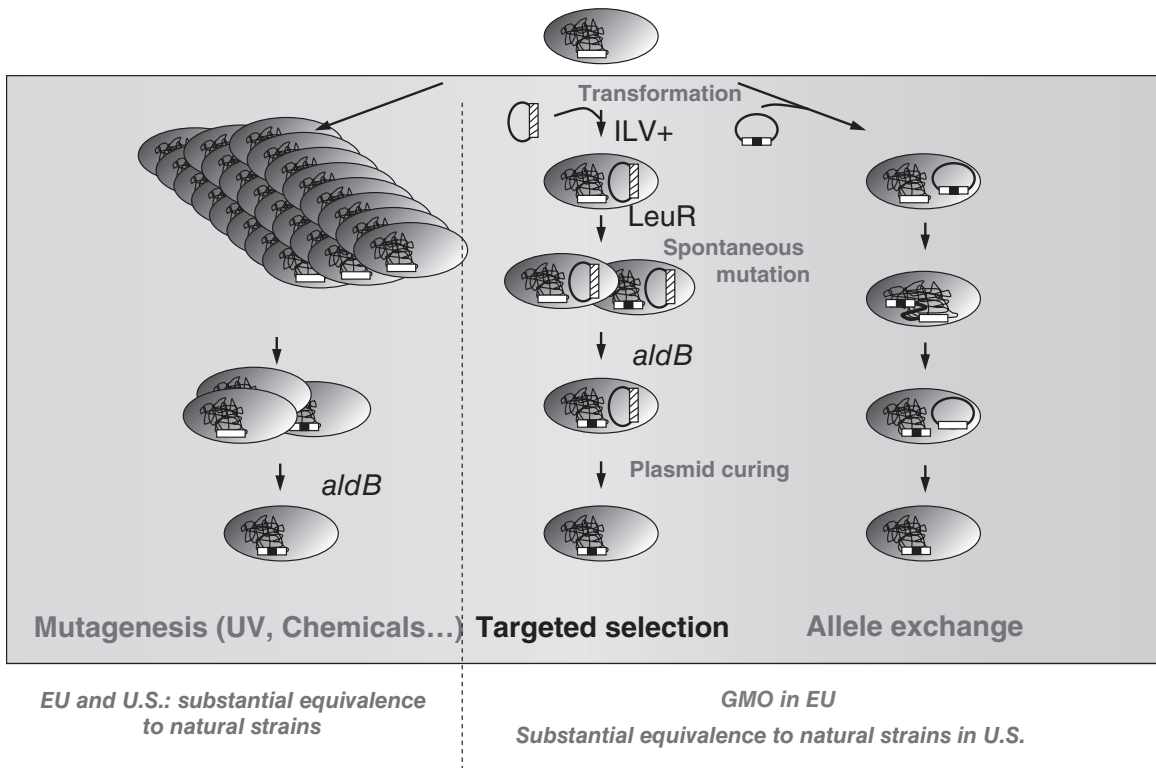
genes and functions originated from different species.

#### 20.4.2. Risk Assessment Procedures

*“Natural” or nongenetically modified bacteria.* In Europe, there is no regulation yet concerning the use of new indigenous strains of microorganisms belonging to species already used in food. Each

country has a regulatory frame that may differ significantly. For example, starter strains may be considered as food ingredients, and therefore indicated on labels as such, or as processing aids, which do not require any labeling on the final product. Many countries have defined lists of safe microorganisms for species that are commonly found in foods (Giraffa 2002) but may also be isolated from clinical cases, such as *Enterococcus* species (Franz et al.





**Figure 20.4.** Example for the difference of GMO regulation in the EU and United States. The same type of mutation in the *aldB* gene can be obtained by different techniques. Resulting strains are considered substantially equivalent in the US regulation and are therefore not submitted to any particular assessment. On the contrary, in Europe, strains where DNA was introduced by a transformation step are systematically considered GMO and submitted to GMO specific regulations. Only the strains obtained by classical mutagenesis are considered substantially equivalent to natural strains.

2001; Wisplinghoff et al. 2004). Among the most well-known Generally Recognized as Safe microorganisms lists, the U.S. list proposes an inventory of microorganisms that can be added in food. Europe is now working on an approach, called Qualified Presumption of Safety, which will propose a list of microorganisms acceptable in food use. On the other hand, microorganisms belonging to new species are subject to “novel food” regulations, which are based on case by case studies found on current scientific knowledge. It is also possible to propose the acceptance of new microorganisms based on the “substantial equivalence” principle. In case of insufficient data, a risk assessment procedure is required that possibly includes toxicity tests or other assessments.

*Genetically modified bacteria.* The regulations for bacteria defined as GMO differ between countries since the definition of GMO differs as well. Food bacteria constructed by “classical genetic methods” (e.g., chemical mutagenesis) are not submitted to any assessment in both Europe and the United States (Fig. 20.4).

GMO risk assessment for food is defined in the 2001/1831/EC and the 97/35/EC recommendations in Europe. In addition to specific points on GMO, this risk assessment shares many aspects with the evaluation of new food processes by novel food. Risk assessment specific for LAB GMO designed for therapeutic purposes are not dealt with in this chapter.

For food GMO, as for new processes in novel food regulation, the following information is required: (1) a detailed description of the new process and its specificities; (2) the origin of the microorganisms used; (3) the effect of the genetic modification, in particular those leading to phenotypical changes; (4) measurements of expression of the new or modified genes; (5) a description of genetic stability of the GMO, in particular if the modification may trigger genomic rearrangements that could modify the properties of the microorganism; (6) the transfer risk of the modified genes; (7) the survival and colonization capacity of the GMO compared with that of the wild type; and (8) the market for the GMO-containing product and expected extent of consumption. All information dealing with any effect of the product on humans and/or the environment has to be provided, in particular, the nutritional value and eventual microbiological and toxicological risks. The potential toxicity and allergenicity resulting from the expression of the new genes should also be clearly mentioned. Toxicity might be due, for example, to the production of new metabolites or their derivatives. The allergenicity might be the result of the production of a new protein. Tests to assess these issues can be relatively simple, but may include, when necessary, animal trials.

#### 20.4.3. *Labeling and Consumer Attitude*

Labeling of GMO-containing products differs in each country. In United States, products containing GMO are not considered significantly different from other new products. In particular, if the GMO-containing product is “substantially equivalent” to any marketed product, it can be commercialized without any specific indication. If relevant differences exist, manufacturers are required to indicate the product’s nutritional value, potential toxicity, or allergenicity. In countries like New Zealand and Australia, the presence of GMO in a product should be included in the list of ingredients.

In Europe, the regulation is much stricter as any product containing GMO or its derivative requires a specific labeling. For example, a spontaneous

diacetyl-overproducing mutant, isolated by using plasmid transformation and curing experiments, is commercialized in the United States but not in Europe (Pedersen et al. 2005). Conversely, mutants in the same gene but isolated by using chemical mutagenesis can be commercialized without any assessment and specific labeling in both, the United States and EU.

## 20.5. **Conclusions**

Knowledge of the genetics of LAB allows the design of modified strains with improved properties compared with wild-type strains. However, their use worldwide might be restricted due to the different countries’ regulatory issues. One question is whether or not it is justified to “label” as GMO strains that have been constructed with DNA of the same species. The basis of this discussion relies on the way “a GMO” is defined, as the result of new techniques (European view) or if only the final result is to be considered (U.S. view). Organisms isolated in nature or modified in laboratories are not systematically considered substantially different that they require specific regulations. Regulatory issues are then focused on the assessment of introduced phenotypes due to the modification, independently of the technique employed.

Regulation divergences may have important commercial consequences, but they may also impact on GMO research, especially in Europe. At the level of trading, the United States estimated that regulation on GMO had to be associated with scientific facts and that, on the contrary, it could be considered non-trade barriers (Kinderlerer 2003). European regulation probably considers the action of anti-GMO movements and the fact that European opinion is mostly not favorable to GMO being introduced in food, may be due in part to mistrust toward the communication of several GMO-producing companies (Barnes 2003; Borch et al. 2003).

Several scientific issues raised during GMO debates have been studied in-depth. For example, post-genomic methods allowed global measurement of the effect of modifications on the metabolisms and gene expression in microorganisms (Renault

2002; Pedersen et al. 2005) and vegetables (Cellini et al. 2004; Kuiper et al. 2004; El Ouakfaoui and Miki 2005). In particular, it has been shown that molecular techniques are not less reliable than traditional modification techniques. Their use does not lead to particular secondary effects (El Ouakfaoui and Miki 2005; Pedersen et al. 2005). Regulation will probably evolve to take into account new scientific data, as well as possible changes in public perception of GMO, which are now widely used in countries such as the United States, China, and several other countries.

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